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TITLE: Deconstruction of Oncogenic K-RAS Signaling Reveals Focal Adhesion Kinase as a Novel Therapeutic Target in NSCLC

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14. ABSTRACT About 25% of lung adenocarcinomas express mutant KRAS (KM) often is association with co-occurring mutations that inactivate the the CDKN2A locus, which comprises p16 ^{INK4A} and p14 ^{ARF} , or the p53 tumor suppressors. These mutations contribute to disease progression. There are no therapies that target cancers that express mutant KRAS. Thus, it is notable that with this grant we found that: 1. Silencing, pharmacologic inhibition or genetic ablation of FAK causes cell death specifically in KM lung cancer cells (KMLC) that are either CDKN2A or p53 mutant; 2. pharmacologic inhibition of FAK causes the regression specifically of high-grade mutant Kras;Cdkn2a null lung cancers in genetically engineered mice; 3. Genetic ablation of FAK in mouse models of KMLC significantly impairs KM growth; 4. FAK silencing, ablation of pharmacologic inhibition impairs the DNA damage response, potentiating the toxic effects of ionizing radiation in preclinical lung cancer models; 5. The SUMO E3 ligase PIAS1 interacts with FAK, promoting its nuclear translocation, oncogenic properties and DNA repair activity. These findings provided the rationale for a multi-center Phase II clinical trial using the FAK inhibitor (FAKi) VS-6063 in KMLC patients (PI Dr. Gerber at UT Southwestern Medical Center). Taken together these data support the conclusion that FAK is a therapeutic target in KMLC.					
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1. Introduction:

This application rests on the hypothesis that the genotype of cancer cells determines specific vulnerabilities that can be exploited in cancer therapy. The KRAS proto-oncogene is a small guanosine triphosphatase that is constitutively activated (mutant KRAS, KM thereafter) in 25% of non-small cell lung cancers (KM lung cancer, KMLC thereafter). There are no drugs that effectively target KMLC. With preliminary experiments, we identified focal adhesion kinase (FAK) as an essential requirement for the survival of KMLC that is deficient for either the CDKN2A or the p53 tumor suppressors. We proposed to test the hypothesis that FAK is a critical druggable vulnerability in KMLC using an approach that integrates the use of lung cancer cell lines, engineered mouse models of KMLC, pharmacologic and genetic means to inactivate FAK. With this research project, we have demonstrated that: 1. silencing, pharmacologic inhibition or genetic ablation of FAK causes cell death specifically in KM lung cancer cells (KMLC) that are either CDKN2A or p53 mutant; 2. pharmacologic inhibition of FAK (FAKi) causes the regression of high-grade mutant Kras;Cdkn2a null lung cancers in genetically engineered mice; 3. genetic ablation of FAK in mouse models of KMLC significantly impairs KM growth; 4. FAK silencing, ablation or treatment with FAKi impairs the DNA damage response, potentiating the toxic effects of ionizing radiation in preclinical KMLC cellular and mouse models; 5. the SUMO E3 ligase PIAS1 interacts with FAK, promoting its oncogenic and DNA repair activity. These findings provided the rationale for a multi-center Phase II clinical trial using the small molecule FAKi Defactinib in KMLC patients (PI Dr. Gerber at UT Southwestern Medical Center). This trial, which is not directly funded by this award, has completed its target accrual of 55 patients (ClinicalTrials.gov Identifier: NCT01951690). This trial showed that single agent treatment with the FAKi Defactinib in a population of heavily pretreated patients, causes tumor stabilization or shrinkage in 25% or patients. This grant led to several publications in high impact journals and to presentations at national and international meetings.

Taken together these data support the conclusion that FAK is a therapeutic target in KMLC and that further clinical testing should be pursued in association with DNA damaging agents or radiotherapy.

2. Keywords

KRAS, lung cancer, mouse lung cancer models, FAK, SUMOylation, PIAS1, targeted kinase inhibitors, Defactinib, RNAi, preclinical studies, radiotherapy, radiosensitization.

3. Accomplishments for grant W81XWH-12-1-0210.

3.1 What were the major goals of the project?

Goal 1. Determination of the anti-cancer effects of FAK inhibition in NSCLC cells. We proposed to use genetic and pharmacologic approaches to establish whether FAK is required for the survival of lung cancer (LC) expressing mutant KRAS (KM lung cancer, KMLC) and deficient for either CDKN2A or for p53 tumor suppressors. We proposed to: 1. Characterize the anti-tumor effects of FAK inhibition in LC; 2. Determine the effect of genetic inactivation of FAK in KMLC using a conditional knock-out allele of FAK.

Goal 2. Identification of strategies that synergize with inhibition of FAK to induce the death of NSCLC expressing oncogenic KRAS. We proposed to use a genetic and pharmacologic approach in LC cells and in mouse lung cancer models to identify strategies to maximize cancer cell death upon pharmacologic inhibition of FAK. We propose to: 1. Determine whether FAK inhibition synergizes with inhibition of PI3K/mTOR signaling or of other druggable oncogenic signaling pathways in LC cells; 2. Complete the validation of a whole genome siRNA screening to identify synthetic lethal interactions in KMLC cells.

For the purpose of this final progress report we will follow the format mandated by the “technical reporting requirements” applied to the approved statement of work.

3.1 What was accomplished under these goals?

Goal #1. Determination of the anti-cancer effects of FAK inhibition in NSCLC cells.

Our preliminary data indicated that FAK is required for the viability of KMLC cells deficient for either CDKN2A or p53 (1). We proposed: 1. To establish whether NSCLC cells are dependent on FAK; 2. To identify the mechanisms responsible for this dependency; 3. To identify additional KM vulnerabilities.

Our preclinical data led to a phase II clinical trial with the FAK inhibitor VS-6063 (ClinicalTrials.gov Identifier: NCT01951690) in KMLC patients. This trial is not directly funded by this grant and has completed its targeted accrual of 55 patients. Our goal was to provide the framework for the development of FAK inhibitors in KMLC.

Subtask 1a. Pharmacologic inhibition of FAK in human NSCLC cells in vitro.

Major activity: We tested the sensitivity of a panel of lung cancer cells (21 cell lines), which include the major mutations that occur in lung cancer, to FAK inhibitors (FAKi) VS-6063 (Defactinib), VS-4718 (previously known as PND- 1186) (2, 3).

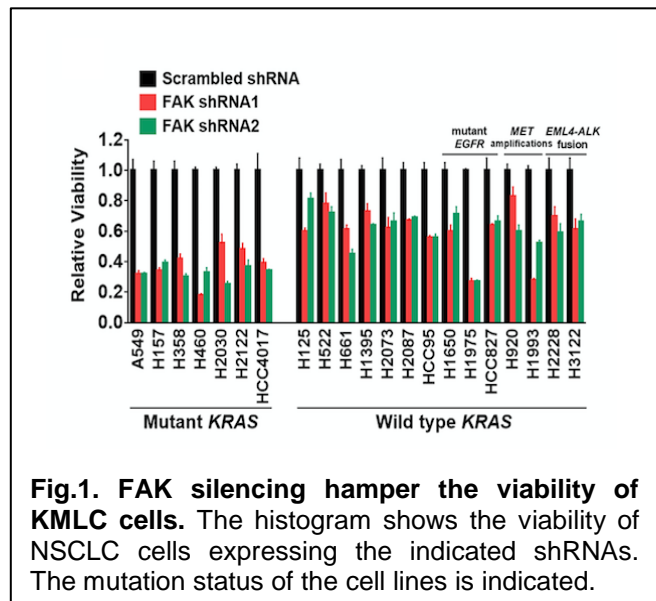
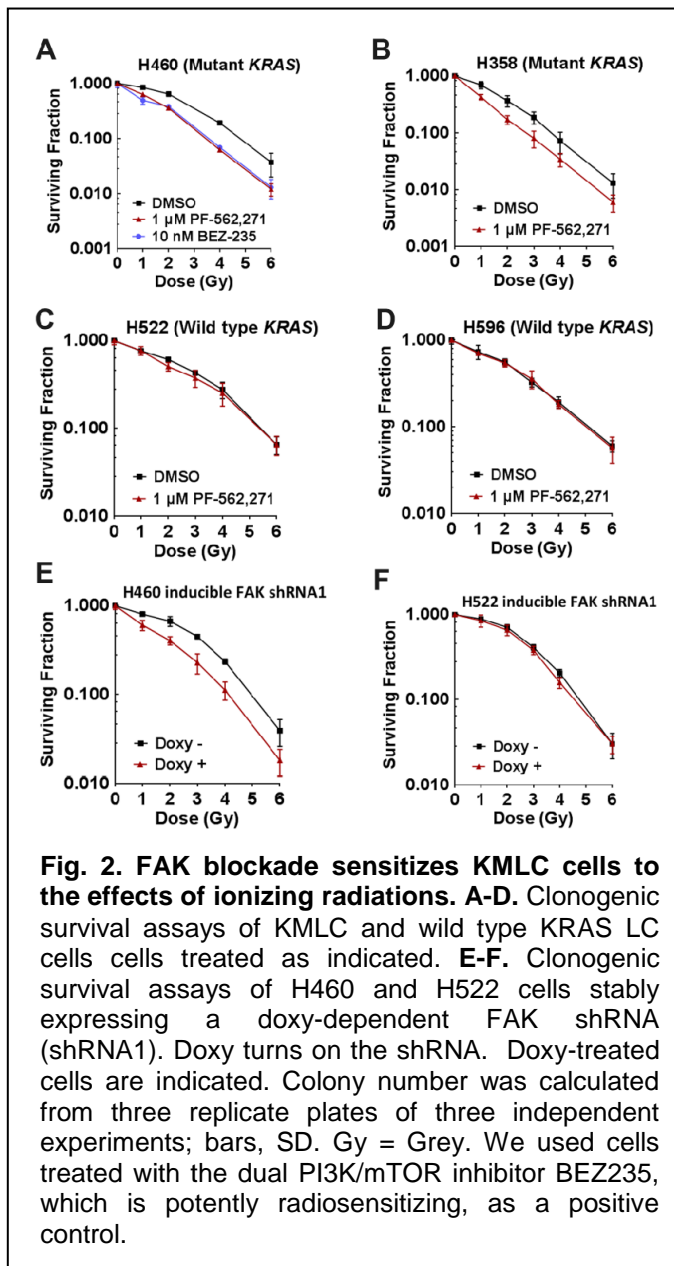


Fig.1. FAK silencing hamper the viability of KMLC cells. The histogram shows the viability of NSCLC cells expressing the indicated shRNAs. The mutation status of the cell lines is indicated.

In aggregate our experiments demonstrated that FAKis lead to significant cytopathic effects in KMLC cells deficient for either CDKN2A or p53. In contrast, FAK silencing did not consistently affect the viability of LC cells carrying genotypes other than KM. For instance, FAK silencing strikingly reduced the viability of H1975 (mutant EGFR) and H1993 (MET amplified) cells, but not of H1650 and HCC827 (mutant EGFR) or H920 cells (MET amplified). We also noticed that the vulnerability to FAK silencing was comparable between mutant KRAS cells that carry p53, CDKN2A, or LKB1 mutations (Fig. 1 and data not shown). Thus, we did not find any oncogenic mutation other than KM that identifies LC cells that are vulnerable to FAKi.

These studies lead to the conclusion that KM is a biomarker that identifies dependency on FAK and accordingly vulnerability to FAKi. We published our findings (1, 4)

Subtask 1b: Determination of the functional consequences of FAK inhibition in NSCLC cells.



Major activity: as proposed in the statement of work, we performed experiments in cultured LC cells.

Specific Objectives: The goal of subtask 1b was to identify the cellular networks that mediate the antitumor effects of FAK suppression in KMLC.

Significant results: We found that in LC cells, FAK inhibition does not affect canonical FAK-dependent signaling pathways (i.e. AKT, ERK or JNK). Instead, we found that FAK silencing or FAKi treatment induces DNA damage. We also found that FAK physically interacts with the SUMO E3 ligase Protein inhibitor of activated STAT1 (PIAS1). This finding is coherent with the observation of others that: 1. a fraction of FAK is SUMOylated and interacts with PIAS1 in the nucleus (5); 2. PIAS1 positively regulates DNA double stand break repair (6). Accordingly, we determined that FAK silencing or FAKi treatment sensitizes KMLC cells to the cytopathic effects of ionizing radiations (4).

For instance, clonogenic survival assays, which are the gold standard to determine the susceptibility to ionizing radiations, demonstrated that FAKi treatment causes radiosensitization of KMLC and not KL cells (Fig 2). For this experiment we exposed to increasing doses of IR (1-6 Gy) H460 and H358 (KMLC) and H522 and H596 cells (LC cells carrying wild type KRAS) as representative examples of LC cells we used in task 1a, (Fig. 2A-D). We administered 1 μM of FAKi PF-562,271 four hours before exposure to ionizing radiations (IR). We chose this concentration because it inhibits the activated form of FAK (phospho-FAK, P-FAK thereafter) in a comparable manner in all the LC cells used for this study (data not shown). We limited the incubation time to 48 hours not to affect cell

plating efficiency. We scored colonies of >50 normal appearing cells 15-30 days after treatment and graphed the survival fraction (SF) versus dose of IR (expressed in Greys, Gy) used. We calculated Do (relative dose of IR required for 37% lethality on a log-phase kill curve), Dq (inherent DNA repair capacity: dose (Gy) required to eliminate the survival curve shoulder) and dose enhancement ratios (DERs at LD₅₀ and LD₂₀) as described (7).

We found that pharmacologic inhibition of FAK resulted in profound changes in Dq as well as significant, but less dramatic, decreases in Do in KMLC cells ((Fig. 2A-B). Thus, we concluded that FAKi reduces inherent DNA repair capacity (Dq). In contrast, exposure of wild-type LC cells to FAKi had no significant effect on IR-induced lethality as noted by the survival curve and estimations of Dq and Do (Fig. 2C-D). Most importantly, the presence of FAKi increased dose enhancement ratios (DERs) with values ranging between 2.1 and 1.9 at LD₅₀ levels, and between 1.7 and 1.4 at LD₂₀ levels, respectively in KMLC cells (Table 1).

Notably, the effect of FAKi treatment was comparable to the dual PI3K/mTOR inhibitor BEZ-235, which is a known radiosensitizing agent (Fig. 2A) (8, 9).

We confirmed these findings with an independent approach. We used the GEPIR retrovirus to express FAK shRNA1, which we validated in preliminary experiments, in a doxycycline-regulated manner at the time of IR exposure (Fig. 2E-F). These findings suggest that **FAK blockade sensitizes KMLC cells to the effects of ionizing radiations**.

Next, we determined that treatment with FAKi PF-562,271 in combination with IR (2 Gy) leads to a

	Dq (Gy)	Do (Gy)	DER Values	
			LD50	LD20
H460 (Mutant <i>KRAS</i>)				
DMSO	2.0	2.0		
PF-562,271	1.0	1.7	1.9	1.6
BEZ-235	1.0	1.7	2.0	1.6
H358 (Mutant <i>KRAS</i>)				
DMSO	1.1	1.4		
PF-562,271	0.2	1.1	1.9	1.7
H522 (wild-type <i>KRAS</i>)				
DMSO	1.4	1.7		
PF-562,271	1.4	1.7		
H596 (wild-type <i>KRAS</i>)				
DMSO	1.7	1.0		
PF-562,271	1.7	1.0		
H460 inducible FAK shFAK1				
Doxy -	2.0	2.1		
Doxy +	1.3	1.0	2.1	1.4
H522 inducible FAK shFAK1				
Doxy -	1.8	2.0		
Doxy +	1.8	1.9		

Table 1. Dq, Do and DER of lung cancer cells treated as indicated. DER at LD₅₀ and LD₂₀ levels were calculated by direct examination of survival curves. Survival parameters (i.e., Dq and Do) were also derived from survival curves shown in Fig. 1 using the relationship, $\log_e n = Dq/Do$. **Note that the effects occur specifically in KMLC cells.**

striking persistence of γ -H2AX foci, a well-known marker of DNA double-strand breaks damage at 24 hours post-IR administration compared with exposure with IR alone. The effect of PF-562,271 was comparable to the effects of the dual PI3K/mTOR inhibitor BEZ-235, a known radiosensitizer (Fig. 3A) (8, 9). Inhibition of FAK in the absence of IR induces γ -H2AX foci slightly above background level in this assay (Fig. 3).

We obtained equivalent results when we determined the induction and resolution of γ -H2AX and TP53BP1 foci, as readout of DNA damage, in H460 (derived from KMLC) cells where we ablated FAK by CRISPR/CAS9 editing (data not shown).

Next, we confirmed the effects on γ -H2AX by flow cytometry (data not shown). In addition, we found that KMLC cells treated with FAKi in association with IR display a significant increase of the percentage of cells in the G₂ phase of the cell as compared to H460 cells treated with IR only (data not shown).

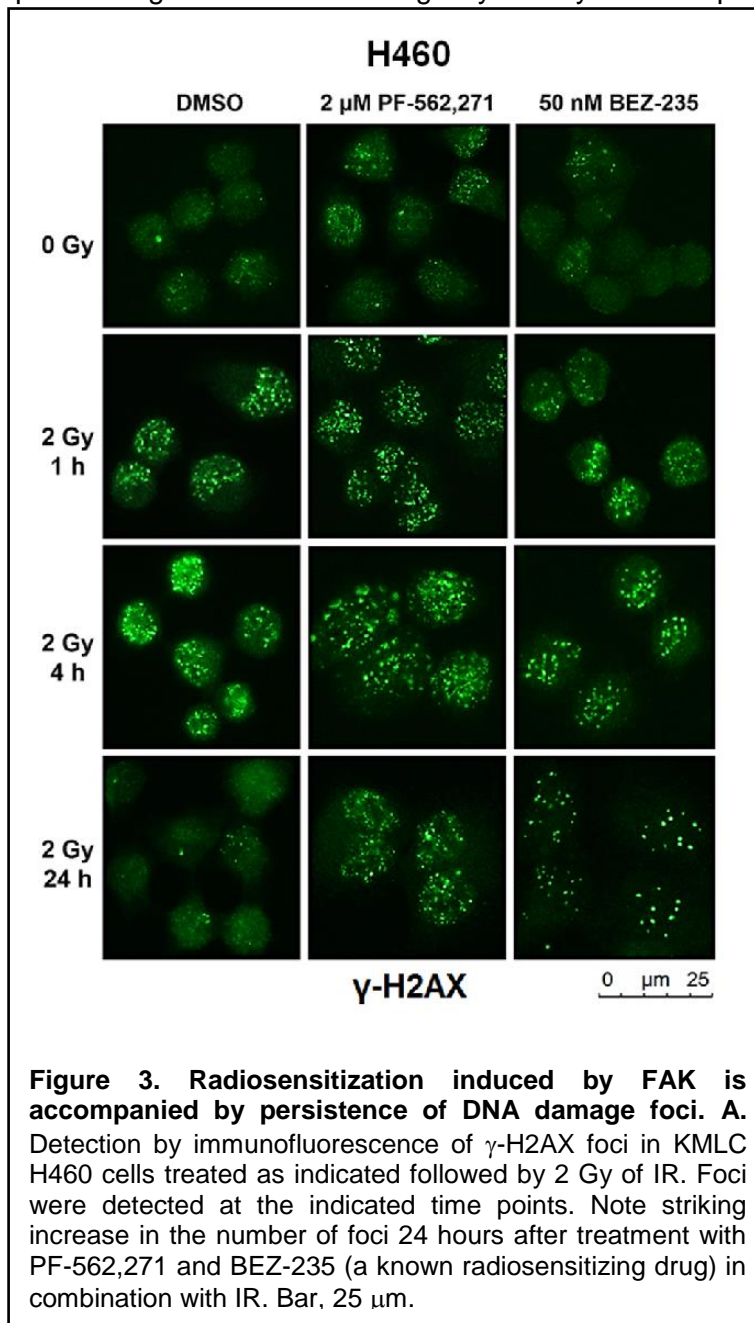
Taken together these data suggest that inhibition/suppression of FAK results in persistent DNA damage in KMLC cells because of inhibition of DNA repair or augmentation of damage by cell cycle checkpoint abrogation, which occur without affecting the activation and recruitment to sites of DNA damage of the DNA damage sensing machinery. These observations also suggest that IR therapy could be exploited to sensitize KMLC to therapy with FAKis.

We reported these findings in the literature (4).

Other achievements:

Using single nucleotide polymorphism (SNPs) data, we discovered that the Small ubiquitin-like modifier (SUMO) E3 ligase PIAS1 and FAK are frequently co-amplified in LC specimens. We also found a positive correlation between increased gene copy number and FAK and PIAS1 protein levels in KMLC cell lines, human LC samples and in a mouse model of metastatic KMLC. SUMO proteins have recently gained attention because of their participation in the covalent modification of target protein substrates, a process referred to as SUMOylation. This process consists of an enzymatic cascade whereby SUMO proteins are added onto target substrates with the involvement of E1, E2 and E3 SUMO ligases. SUMOylation has been implicated in several cellular processes that include the regulation of nuclear import, DNA damage repair and signal transduction.

These observations prompted us to characterize the relationship between PIAS1 and FAK in KMLC. This is a brief summary of our results: 1. *PIAS1* and *FAK* genes are co-amplified in LC cell lines; 2. FAK and PIAS1 proteins are upregulated in metastatic LC; 3. PIAS1 and FAK colocalize in the perinuclear region ; 4. FAK knockdown leads to significant reduction of PIAS1 protein and vice versa; 5. PIAS1 silencing impairs several



of the oncogenic functions that FAK exerts in KMLC cells, including DNA repair of double strand breaks. We reported these findings in the literature (10).

Subtask 1c: Pharmacologic inhibition of FAK in human NSCLC cells in vivo.

Major activity: Treatment with FAKi mice carrying xenografts of lung cancer cell lines.

Specific Objectives: Testing the antitumor effect of pharmacologic inhibition of FAK in human lung cancer cells grown as xenografts in mice.

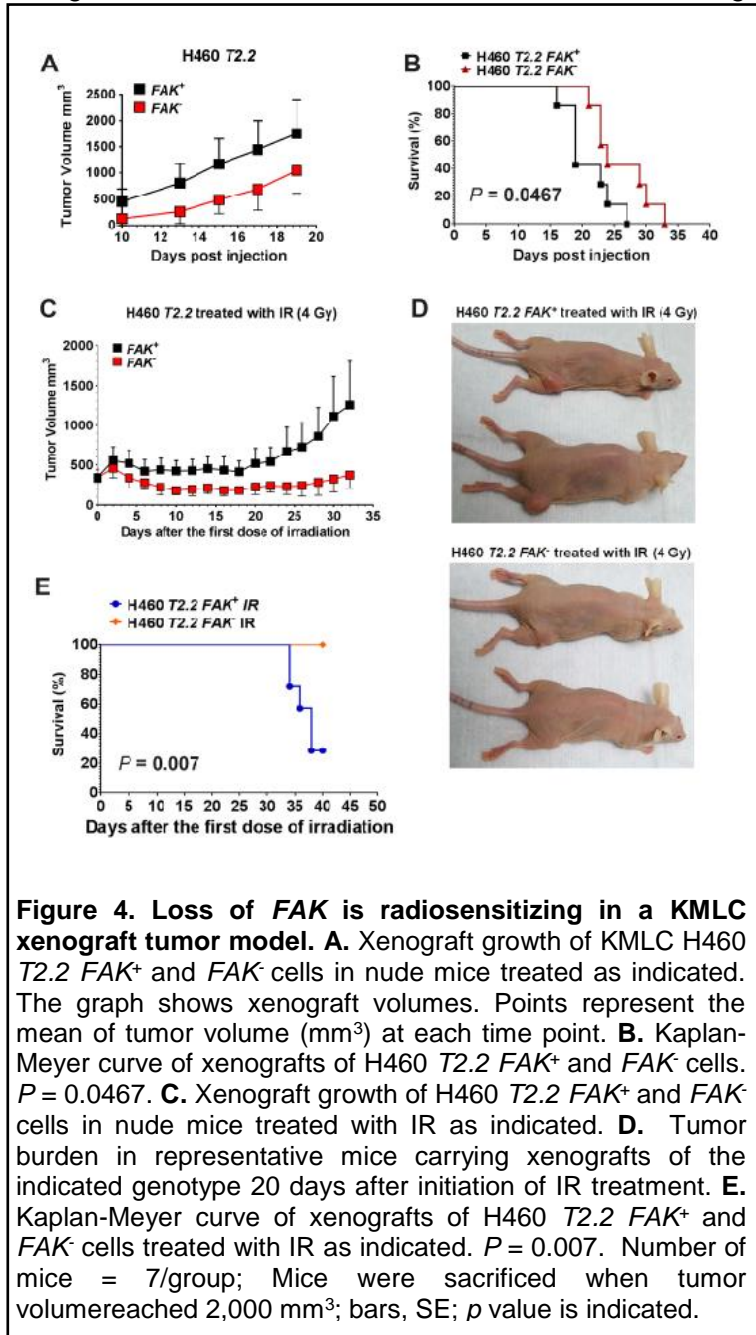
Significant results: We tested the anti-tumor effects of FAK inactivation mediated by CRISP/CAS9 editing, which was not available at the time the initial grant was submitted. In agreement with the data

shown in Figure 1, we found that ablation of FAK significantly decreased the growth or H460 and H358 KMLC cells, increasing also the survival of mice carrying FAK null xenografts (Fig. 4A-B and data not shown). We obtained equivalent results in xenografts of KLMC cells treated with FAKi. These findings indicate that FAKi, as many of the anticancer drugs available today, may have limited efficacy as single agent in cancer therapy.

Thus, we tested whether FAK ablation results in meaningful radiosensitization in KMLC vivo. To this end, we generated cohorts of athymic nude mice bearing xenografts of H460 T2.2 FAK⁺ and H460 T2.2 FAK⁻ cells. When xenografts reached 300 mm³ in size, we delivered five 4 Gy fractions every other day for 10 days specifically to the tumor xenograft using lead-shielded mice. We used a fractionated dose to limit overall tissue toxicity and mimic the administration modality used in the clinic (11). Notably, this xenograft volume and IR dose is comparable with previous studies involving H460 xenografts (8, 12).

IR treatment of H460 T2.2 FAK⁻ cells resulted in a greater than 75% reduction in xenograft volume as compared to H460 T2.2 FAK⁺ cells 30 days after the first dose of IR ($P < 0.001$) (Fig. 4C-D). All irradiated mice carrying xenografts of H460 T2.2 FAK⁻ cells were alive 40 days after the initiation of IR treatment, in contrast five out of seven mice carrying of H460 T2.2 FAK⁺ cells were sacrificed between days 34 and 38 post-radiation due to excessive tumor burden (Fig. 4E).

IR treatment was well tolerated in xenograft bearing nude mice and we didn't



observe any drop in body weight or other signs of toxicity in both groups (data not shown).

These results indicate that the ablation of FAK leads to significant radiosensitizing effects, which in turn led to significant anti-tumor effects *in vivo*. We reported these results in the literature (4).

Other achievements: NA

Subtask 1d. Determination of anti-tumor effects due to pharmacologic inhibition of FAK in transgenic mice with KMLC.

Major activity: Treatment with FAKi of genetically engineered mice with KMLC.

Specific Objectives: Testing the antitumor effect of FAKi *in vivo* in mouse models of KMLC.

Significant results: We have determined that FAK inhibition causes striking inhibition of lung adenocarcinomas but not of lung adenomas of mutant *KRAS/Cdkn2a*^{-/-} mice (1). Thus, we proposed to determine whether FAKi increases overall survival. However, the results of task 1c, reduced the enthusiasm for these experiments because they suggest that human KMLC cells have mechanisms that allow the bypass of FAK loss. Indeed, lung cancers invariably develop resistance to treatment with targeted cancer drugs. Exactly to counteract this concern, we proposed to test FAKi in combination with other agents (Task 1c).

Other achievements: NA

Subtask 1e. Determination of the impact of FAK deficiency in transgenic mice with KMLC.

Major activity: Study the impact of Fak deficiency on KMLC in genetically engineered mice.

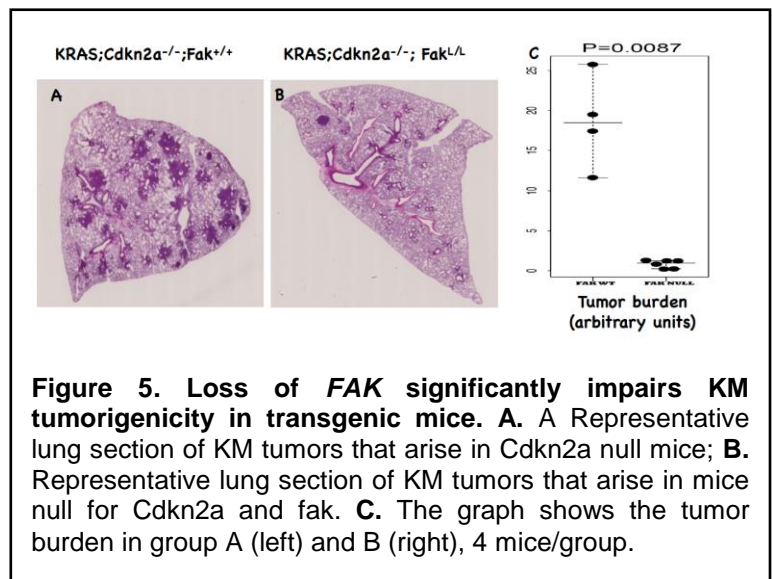
Specific Objectives: determination of the impact of Fak deficiency on mutant Kras tumorigenesis *in vivo* in genetically engineered mice.

Significant results: We proposed to test the requirement of Fak for the progression from KM lung adenoma to adenocarcinoma. For this purpose, we are using mice carrying a floxed allele of *Fak* to generate cohorts of *LsL-Kras/Cdkn2a*^{-/-} and *LsL-Kras/Cdkn2a*^{-/-}/*Fak*^{fl/fl} mice (13). In this mouse model, KM is activated by the delivery of the cre recombinase to the respiratory epithelium with a recombinant adenovirus (adeno-Cre) (14).

We found a striking reduction in KMLC burden in *LsL-Kras/Cdkn2a*^{-/-}/*Fak*^{fl/fl} mice as compared to *LsL-Kras/Cdkn2a*^{-/-} mice (Fig 5). As expected, genotyping of microdissected tumors of *LsL-Kras/Cdkn2a*^{-/-} confirmed that adeno-cre excised *Cdkn2a* (not shown). The striking reduction in KMLC foci, indicates that Fak is essential for the initiation of KMLC *in vivo*, establishing FAK as a high value therapeutic target.

Goal #2. Identification of strategies that synergize with inhibition of FAK to induce the death of NSCLC expressing oncogenic KRAS.

We proposed to use genetic and



pharmacologic approaches to identify strategies to optimize the therapeutic outcome of inhibition of FAK in KMLC cells mutant for either CDKN2A or p53.

Subtask 2a. Identification of combination therapies that synergize with pharmacologic inhibition of FAK;

Major activity: testing the effects of drug combinations in cultured lung cancer cells.

Specific Objectives: identification of combination therapies that synergize with inhibition of FAK.

Significant results: We prioritized our efforts to determine whether FAK inhibitors synergize with inhibitors of proteins that interact with FAK, such as c-MET, EGFR, SRC, PI3K, MEK1/2 or mTORC1/2. We have not identified synergistic relationships, but only additive relationships. This finding may indicate that in KMLC cells, FAK signals through non-canonical pathways. This hypothesis is consistent with the observation that FAK silencing/inhibition leads to radiosensitization (refer to subtask 1b). This finding reveals a novel and unexpected function of FAK, which could be harnessed for therapeutic purposes in KMLC.

Other achievements: NA

Subtask 2b. Complete the validation of a whole genome siRNA screening to identify oncogenic KRAS synthetic lethal interactions in NSCLC cells.

Major activity: Perform experiments to identify genes that when inactivated cause the death of mutant KRAS lung cancer cells .

Specific Objectives: Identification of additional mutant KRAS synthetic lethal interaction that could represent therapeutic targets.

Significant results: We completed the validation of the whole-genome synthetic lethal siRNA screening aimed at identifying genes that when are silenced induce cell death in mutant KRAS cells treated with the PI3K/mTOR inhibitor BEZ235 used at a 200 nM concentration (15). For the primary screening we used HCC44 KMLC cells (mutant KRAS; mutant p53). At this concentration BEZ235 causes more than 80% inhibition of mTORC1/2 signaling, but less than 20% inhibition of cell viability. In the primary screening we compared the viability of HCC44 cells treated with control siRNA and 200nM BEZ235 to the viability of cells treated with siRNAs targeting individual genes tested

	H2087	H460	HCC44	H23	H1819	A549
THBS3	y	y	y	y	y	n
HNRNP2	n	y	y	y	n	y
GPX4	n	n	y	n	n	n
RRAGD	n	y	y	y	n	y
NDRG1	n	n	y	n	n	n
G22P	n	y	y	n	n	y
TBK1	n	y	y	y	n	y
WNK1	y	y	y	y	n	y
TBC1D14	NT	NT	y	NT	y	y

Table 2. The table shows the results of the secondary screening to identify genes that when silenced co-operate with the PI3K/mTORC1/2 inhibitor BEZ235 in causing death of lung cancer cells. Cell lines are indicated on the horizontal line. Genes on vertical line. Y (i.e. yes): cooperation between silencing and BEZ235 treatment; N (i.e no): no co-operation was observed. NT; Not tested.

with BEZ235. In parallel, we determined the effects of silencing the targeted genes in cells treated with DMSO (the vehicle of BEZ235).

We considered positive hits genes that when silenced would cause less than 20% inhibition of viability but that would cause more than 70% viability in association with BEZ235. We chose to validate 50 of the top 300 statistically significant hits in HCC44 KMLC cells. We chose genes based on the biological activity and availability for reagents to study their function. We confirmed 9 hits. Next, we determined whether their effects were generalizable to the following cell lines: HCC44, H460, H23 and A549 NSCLC cells as representative examples of mutant KRAS NSCLC cells and in H2087 (wild type KRAS;p53 mutant) and H1819 (no mutations noted in major oncogenes/tumor suppressor genes) LC cells. Table 2 shows the genes that we have validated. RRAGD (Ras-Related GTP Binding D) is a GTP-binding protein implicated in the regulation of mTORC1. This finding suggests that a more complete inhibition of mTORC may have increased cytotoxic effects. TBK1 (TANK-binding kinase 1) has been implicated in transducing pro-survival signals in mutant KRAS lung cancer (16, 17).

The execution and validation of the synthetic lethal screening has been a very time consuming and labor intensive task. We learned that most synthetic lethal interactions occur only to the cell line used for the primary screening. Thus, there is a tremendous attrition of the “hit” that are generalizable across several cell lines. In addition, most of the hits found are not druggable or do not belong to networks that are druggable. Finally, there are issues of reproducibility of RNAi screenings across different platforms. These intrinsic limitations of the RNAi screening were recognized by NCI with the recently released SolveRAS RFA. This RFA invites projects for innovative and improved synthetic lethal screening. For this reason, we feel satisfied that our screening revealed FAK as a bona fide preclinical target. Accordingly, we consider the task of the validation of the synthetic lethal hits completed.

Other achievements: NA

3.2 What opportunities for training and professional development has the project provided?

This project was not specifically intended to provide training and professional development opportunities. Nevertheless, this project was instrumental in providing resources to fund the work of several trainees. Dr. Mahesh Padanad (post-doctoral fellow), Dr. Ke-Jing Tang (Associate Professor at Sun Yat-sen University, Guangzhou, China, Visiting professor), Niranjana Venkateswaran, MS (Research technician) and Jerfiz Constanzo BS (Graduate student) conducted the research described in this progress report. All these trainees published manuscript detailing their work on this project. Their involvement in this project is part of their research training under the mentorship of Dr. Scaglioni, MD.

3.3 How were the results disseminated to communities of interest?

We presented our data at several national and international cancer conferences. In addition, these data were published in the following peer-reviewed publications. Please refer to section 6 for details.

4. Impact.

With this research we have identified FAK as a novel targetable vulnerability of KMLC. Our preclinical data provided the rationale for the design and execution of a Phase II multi-institutional clinical trial in lung cancer patients, which has completed the target enrolment of 55 KMLC patients.

4.1. What was the impact on the development of the principal discipline(s) of the project.

Our research project indicates that KMLCs that are mutant for either CDKN2A or for p53 are vulnerable to FAK silencing or pharmacologic inhibition both in cultured cells and in transgenic mice with high-grade KMLC.

This oncogenotype is of clinical relevance. We have determined that mutant KRAS;CDKN2A deficiency occurs in about 50% of KMLC. Mutant KRAS is present in 25% of the 175,000 newly diagnosed lung cancers that are diagnosed yearly in the USA yearly. Thus, the mutant KRAS;CDKN2A deficient genotype is present in about 20,000 to 25,000 patients/year in the USA. Mutant KRAS;p53 deficiency has a similar prevalence (18, 19).

We also identified a novel function of FAK, namely its ability to promote the repair of DNA damage. Our preliminary data indicate that FAK silencing or pharmacologic inhibition lead to defective DNA double-strand break repair causing significant radiosensitizing effects. Accordingly, FAK inhibition/silencing overcomes radioresistance of KMLC cells cultured in vitro. We are currently pursuing the hypothesis that FAK exerts its effect on the DNA damage repair machinery through the SUMO E3 ligase PIAS1.

Our data provided the rationale for the execution of the first clinical trial of a small molecule inhibitor of FAK in lung cancer patients (ClinicalTrials.gov Identifier: NCT01951690). This is a multicenter Phase 2 trial of the FAK inhibitor defactinib (VS-6063) to treat KRAS-mutated non-small cell lung cancer (NSCLC). This trial has completed its targeted accrual of 55 patients. Treatment outcomes will be correlated to the presence of CDKN2A or p53 mutations.

4.2. What was the impact on other disciplines?

Our data indicate that KMLC, which was considered a homogenous disease, can be further sub-classified based on co-occurring mutations and that specific oncogenotypes may dictate response to targeted therapy. It is conceivable that this paradigm may apply to cancer of other histological origins.

4.3. What was the impact on technology transfer?

A phase II multicentrer clinical trial sponsored by Verastem is ongoing (ClinicalTrials.gov Identifier: NCT01951690).

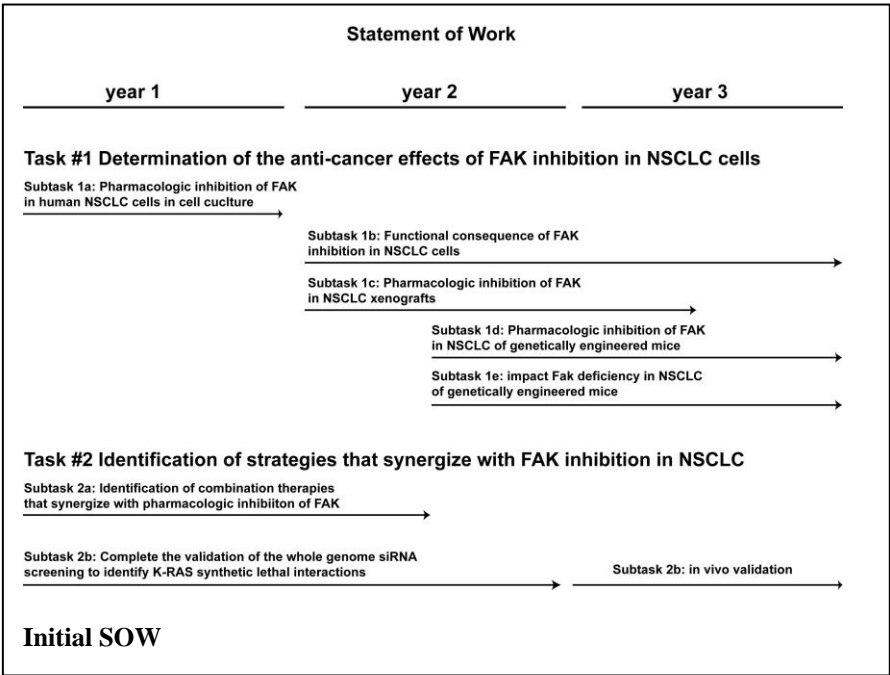
4.4. What was the impact on society beyond science and technology?

None to report

5. Changes/Problems.

We made no changes to the initial SOW.

The scheme of the intial SOW is shown on the right.



6. Products.

6.1. Publications, conference papers, and presentations.

Manuscripts:

1. Schuster K, Venkateswaran N, Rabellino A, Girard L, Pena-Llopis S, Scaglioni PP. Nullifying the CDKN2AB Locus Promotes Mutant K-ras Lung Tumorigenesis. *Molecular cancer research : MCR*. 2014;12(6):912-23. doi: 10.1158/1541-7786.MCR-13-0620-T. PubMed PMID: 24618618; PubMed Central PMCID: PMC4058359.
2. Tang, K.J., Constanzo, J.D., Venkateswaran, N., Melegari, M., Ilcheva, M., Morales, J.C., Skoulidis, F., Heymach, J.V., Boothman, D.A., and Scaglioni, P.P. (2016). Focal Adhesion Kinase Regulates the DNA Damage Response and Its Inhibition Radiosensitizes Mutant KRAS Lung Cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 22, 5851-5863.
3. Constanzo, J.D., Tang, K.J., Rindhe, S., Melegari, M., Liu, H., Tang, X., Rodriguez-Canales, J., Wistuba, I., and Scaglioni, P.P. (2016). PIAS1-FAK Interaction Promotes the Survival and Progression of Non-Small Cell Lung Cancer. *Neoplasia* 18, 282-293.

Abstracts at national and international meetings:

1. G. Konstantinidou, G. Ramadori, F. Torti, C. Behrens, I. I. Wistuba, A. Heguy, D. Gerber, J. Teruya-Feldstein, P.P. Scaglioni. Identification of Focal Adhesion Kinase (FAK) as a Therapeutic Target in KRAS Mutant Lung Cancer. *Mechanisms and Models of Cancer*. Salk Institute, La Jolla, CA. August 7-10 2013. **Note that this abstract was selected for oral presentation.**
 2. K. Schuster, N. Venkateswaran, S. Peña-Llopis and P.P. Scaglioni. Loss of the CDKN2AB Tumor Suppressor Locus Promotes Mutant KRAS Lung Tumorigenesis. *Mechanisms and Models of Cancer*. Salk Institute, La Jolla, CA. August 7-10 2013.
 3. J.D. Constanzo, A. Rabellino, G. Konstantinidou, K. Schuster and P.P. Scaglioni. SUMO Ligase PIAS1: An Unexpected Role In Cancer Cell Initiation And Survival. *Mechanisms and Models of Cancer*. Salk Institute, La Jolla, CA. August 7-10 2013.
 3. P.P. Scaglioni. Identification of Focal Adhesion Kinase (FAK) as a therapeutic target in KRAS mutant lung cancer. Lung Spore Workshop. National Cancer Institute., Rockville, MD. July 11-12, 2013. **Note that this abstract was selected for oral presentation.**
 4. P.P. Scaglioni. Inhibiting Focal Adhesion Kinase in Mutant KRAS NSCLC. Lung Cancer Summit. Ritz Carlton Dallas, Texas 12/15/2013.
 5. K.J. Tang, J. D. Constanzo, N. Venkateswaran, M. Melegari, J.C. Morales, D.A. Boothman, P.P. Scaglioni. Pharmacologic inhibition or genetic ablation of Focal Adhesion Kinase (FAK) is radiosensitizing in KRAS mutant lung cancer. Lung Cancer SPORE Workshop. Rockville, MD. 6/15-6/16 2014. **Note that this abstract was selected for oral presentation.**
- D.E. Gerber, S.S. Ramalingam, D. Morgensztern, R. Kelly, T.F. Burns, A. Lopez-Chavez, A. Wehbe, D.R. Spigel, R. Sorensen, D. Weaver, J. Horobin, M. Keegan, P. P. Scaglioni, D. R. Camidge. A phase 2 study

of defactinib (VS-6063), a cancer stem cell inhibitor that acts through inhibition of focal adhesion kinase (FAK), in patients with KRAS-mutant non-small cell lung cancer. 2014 ASCO Annual Meeting. J Clin Oncol 32:5s, 2014 (suppl; abstr TPS8126).

K.J. Tang, J.D. Constanzo, N. Venkateswaran, M. Melegari, J.C. Morales, D.A. Boothman, **Scaglioni P.P.** Pharmacologic inhibition or genetic ablation of Focal adhesion kinase (FAK) is radiosensitizing in mutant KRAS lung cancer. 15th Annual Targeted Therapies of Lung Cancer Meeting. The Fairmont Miramar Hotel, Santa Monica, CA. Sponsored by the IASLC. February 18-21, 2015. **Note that this abstract was selected for oral presentation.**

J.D. Constanzo, K.J. Tang, S. Rindhe, N. Venkateswaran, M. Melegari and P.P. Scaglioni. FAK and PIAS1 Genes Promote the Survival and Progression of Aggressive Metastatic Lung Cancer By Engaging DNA Repair Networks and Mitochondria Metabolism. The 10th Symposium Mechanisms and Models of Cancer. The Salk Institute, La Jolla, CA. August 5-8, 2015.

D.E. Gerber, D.R. Camidge, D. Morgensztern, J. Cetnar, R.J. Kelly, S.S. Ramalingam, D.R. Spigel, W. Jeong, **P.P. Scaglioni**, M. Li, M. Keegan, J.C. Horobin, T.F. Burns. Phase II study of Defactinib, VS-6063, a focal adhesion kinase (FAK) inhibitor, in patients with KRAS mutant non-small cell lung cancer (NSCLC). 16th World Conference on Lung Cancer. Denver, CO. September, 6-9, 2015
Presentations at Academic Institutions. Presenter, Pier Paolo Scaglioni, MD.

Metabolic and Signaling Vulnerabilities of KRAS-driven lung adenocarcinoma. European Institute of Oncology, Milano, Italy. Grand Rounds. 1/22/2013.

Metabolic and Signaling Vulnerabilities of KRAS-driven lung adenocarcinoma. University of California, Division of Hematology/Oncology. Irvine, CA. 1/31/2013.

Metabolic and Signaling Vulnerabilities of KRAS-driven lung adenocarcinoma. Division of Hematology/Oncology. UT Health Science Center. San Antonio, TX. 2/21/2013.

Genotype-Specific Cancer Vulnerabilities: The case of PML-RARA and mutant KRAS. Indiana University Medical Center. Indianapolis, IN. 4/18/2013.

Genotype-Specific Cancer Vulnerabilities: The case of PML-RARA and mutant KRAS. Vanderbilt University Medical Center. Nashville. TN. 5/14/2013.

Genotype-Specific Cancer Vulnerabilities: The case of PML-RARA and mutant KRAS. Case Comprehensive Cancer Center. Cleveland, OH. 5/22/2013.

Genotype-Specific Cancer Vulnerabilities: The case of PML-RARA and mutant KRAS. City of Hope, Duarte, CA. 10/04/2013.

Novel targetable vulnerabilities of mutant KRAS lung cancer. University of Iowa, Carver College of Medicine. 11/30/2016

6.2 Website(s) or other Internet site(s)

NA

6.3 Technologies or techniques

We are generating genetically engineered lung cancer models: we will make them available to the scientific community as detailed in the appropriate section of the funded grant.

6.4 Inventions, patent applications, and/or licenses

NA

6.5 Other Product

NA

7. Participants and other collaborating organizations.

7.1. what individuals have worked on the project?

Name:	Mahesh Padanad
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Execution of Cell biology Experiments
Funding Support:	CPRIT Institutional Training Grant (since 3/2014)

Name:	Ke-Jing Tang
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Planning, execution and interpretation of cell biology experiments
Funding Support:	Visiting Scholar supported by the Sun Yat-sen University, Guangzhou, China.

Name:	Niranjan Venkateswaran
Project Role:	Research technician II
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Management of mouse colony and execution of experiments with mouse lung cancer models
Funding Support:	DOD

Name:	Jerfiz Constanzo
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Planning, execution and interpretation of cell biology experiments
Funding Support:	NIH/NCI T32 training grant

7.1. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The following are the research funds received by Dr. Scaglioni, the PI of this project.

Grant #RP150519

6/1/2016-5/31/2020

2.0 cal. Month

Cancer Prevention and Research Institute of Texas (CPRIT)

Title: "Defining and Defeating Mechanistic Subtypes of KRAS-mutant Lung Cancers".

Major Goal: this research program grant proposes to identify and target vulnerabilities of mutant KRAS that occur in the context of specific single codon KRAs mutations or in the setting of multiple co-mutations, and to better understand how to target these co-mutations. **Role: PI of project 2.**

7.2. What other organizations were involved as partners?

None to report

8. Appendices: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

1. CV of Dr. Pier Paolo Scaglioni.
2. Copy of Publications funded by this research grant.

9. References

1. Konstantinidou G, Ramadori G, Torti F, Kangasniemi K, Ramirez RE, Cai Y, et al. RHOA-FAK is a required signaling axis for the maintenance of KRAS-driven lung adenocarcinomas. *Cancer discovery*. 2013;3(4):444-57. doi: 10.1158/2159-8290.CD-12-0388. PubMed PMID: 23358651; PubMed Central PMCID: PMC3625467.
2. Roberts WG, Ung E, Whalen P, Cooper B, Hulford C, Autry C, et al. Antitumor activity and pharmacology of a selective focal adhesion kinase inhibitor, PF-562,271. *Cancer Res*. 2008;68(6):1935-44. Epub 2008/03/15. doi: 68/6/1935 [pii]10.1158/0008-5472.CAN-07-5155. PubMed PMID: 18339875.
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4. Tang KJ, Constanzo JD, Venkateswaran N, Melegari M, Ilcheva M, Morales JC, et al. Focal Adhesion Kinase Regulates the DNA Damage Response and Its Inhibition Radiosensitizes Mutant KRAS Lung Cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2016;22(23):5851-63. doi: 10.1158/1078-0432.CCR-15-2603. PubMed PMID: 27220963; PubMed Central PMCID: PMC5122471.
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8. Konstantinidou G, Bey EA, Rabellino A, Schuster K, Maira MS, Gazdar AF, et al. Dual phosphoinositide 3-kinase/mammalian target of rapamycin blockade is an effective radiosensitizing strategy for the treatment of non-small cell lung cancer harboring K-RAS mutations. *Cancer Res*. 2009;69(19):7644-52. Epub 2009/10/01. doi: 0008-5472.CAN-09-0823 [pii] 10.1158/0008-5472.CAN-09-0823. PubMed PMID: 19789349; PubMed Central PMCID: PMC2760010.
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10. Constanzo JD, Tang KJ, Rindhe S, Melegari M, Liu H, Tang X, et al. PIAS1-FAK Interaction Promotes the Survival and Progression of Non-Small Cell Lung Cancer. *Neoplasia*. 2016;18(5):282-93. doi: 10.1016/j.neo.2016.03.003. PubMed PMID: 27237320; PubMed Central PMCID: PMC4887597.
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10.1038/nature08460. PubMed PMID: 19847166; PubMed Central PMCID: PMC2783335.
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Appendix

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME Scaglioni, Pier Paolo	POSITION TITLE Associate Professor		
eRA COMMONS USER NAME (credential, e.g., agency login) scagliop			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	MM/YY	FIELD OF STUDY
University of Modena, Italy	M.D.	10/1989	Medicine
University of Modena, Italy	Resident	7/1994	Internal Medicine
Fox Chase Cancer Center	Visiting Scientist	3/1993	Virology
Massachusetts General Hospital	Research fellow	6/1998	Virology
Montefiore Medical Center, Bronx, NY	Resident	6/2001	Internal Medicine
Memorial Sloan Kettering Cancer Center, New York, NY	Clinical Fellow	8/2005	Medical Oncology

A. Personal Statement

I am a medical oncologist and physician-scientist trained in molecular virology and cancer biology. In addition to leading a cancer biology research laboratory, I maintain a clinical practice focused on the treatment of acute leukemias. The lab is interested in understanding how genetic alterations create druggable vulnerabilities. To enable our research, we integrate the use of mouse cancer models, cancer cell lines, small molecule inhibitors, RNAi and CRISPR technologies. We have been working on acute promyelocytic leukemia (APL), acute lymphoblastic leukemia (ALL) and mutant KRAS lung cancer because these cancer types depend on their initiating oncogenes. With our work on acute leukemia, we have found that: 1. the PIAS1 SUMO E3 ligase is oncogenic through its interaction with PML, PML-RARA and MYC; 2. SUMOylation is critical in mediating the therapeutic action of arsenic trioxide in APL; 3. Mitochondrial function is critically involved in the maintenance of Phila+ ALL in patient derived xenografts and in mouse models of ALL. We have also identified several vulnerabilities of mutant KRAS including a novel RHOA-FAK axis, which is essential for the maintenance of mutant KRAS lung cancer. This work led to a clinical trial of a FAKi in lung cancer (NCI identifier NCT01951690). In related work, we found that mutant KRAS reprograms lipid homeostasis establishing a reliance on fatty acids metabolism and β -oxidation.

As a member of the Bone Marrow Transplant Service and Disease Oriented Team and as a Co-PI of the UT Lung cancer SPORE grant, I have long-standing interactions with a network of investigators dedicated to the study of leukemia and lung cancer as well as access to clinical and research databases. In addition, as a co-leader of the Simmons Cancer Center Cell Networks Program, I lead an institutional effort to promote cancer research co-ordinating cancer biologists with clinical investigators. This projects and environment have fostered the development of several trainees, several of which have obtained faculty positions.

B. Positions and Honors**Positions and Employment**

1989-1994 Resident in Internal Medicine, Department of Medicine, University of Modena Medical School, Italy.
Program Director: B. Bonati MD.

1991-1993 Visiting Scientist, Department of Molecular Virology, Fox Chase Cancer Center, Philadelphia, PA.
Research Advisor: C. Seeger, Ph.D.

1993-1998 Research Fellow in Medicine, Massachusetts General Hospital, Boston, MA. Research Advisor: J. Wands, MD.

1998-2001 Resident in Medicine, Montefiore Medical Center, The University Hospital for the Albert Einstein College of Medicine, Bronx, NY. Program Director: Joan Casey, MD.

2001- 2005: Fellow in Hematology/Oncology, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY. Program Directors: D. Bajorin, MD & M. Heaney, MD.

2002-2005: Post-Doctoral Fellow, Department of Pathology and Cancer Biology & Genetics Program, Sloan-Kettering Institute, New York, NY. Research Advisor: Pier Paolo Pandolfi, MD, Ph.D.

2005-2006. Instructor in Medicine, Memorial Sloan-Kettering Cancer, Department of Medicine, Division of Hematologic Oncology, Hematology Service, New York, NY.

2006-2014. Assistant Professor, Department of Medicine, Division of Hematology/Oncology, UT Southwestern Medical Center. Dallas, TX.

2014-Present. Associate Professor with Tenure, Department of Medicine, Division of Hematology/Oncology, UT Southwestern Medical Center. Dallas, TX.

Honors

1989 Medicina Prize for Italian Researchers younger than 35 years of age; 1991 Terme di Chianciano S.p.A. Fellowship; 2001 Montefiore Medical Center/A. Einstein College of Medicine: Citation in recognition as an outstanding House Officer; 2003 ASCO Young Investigator Award; 2003 CALGB Oncology Fellows Award

2003 ASH Travel Award; 2004 Michael and Ethel L. Cohen Fellow; 2004 Charles A. Dana Fellow, Clinical Scholars Training Program in Biomedical Research; 2004 Doris Duke Dinner Award

2004 ASH Travel Award; 2008 Gibbie Award Ryan Gibson Foundation

2012 Gibbie Award Ryan Gibson Foundation; 2014 Texas 4000 for Cancer Award.

Peer review Committees

2013-Present AACR Basic Cancer Research Fellowships Scientific Review Committee

2013-Present NCI Study Section J

Board Certifications

2001 American Board of Internal Medicine

2004 American Board of Medical Oncology

C. Contribution to Science

1. My early work as a post-doctoral fellow focused on the study of the molecular biology of the hepatitis B virus (HBV), a major human pathogen and a cause of hepatocellular carcinoma. I focused my efforts on the study of the mechanisms that govern the assembly and replication of HBV with the goal to identify innovative therapeutic strategies. I identified dominant negative mutants of the HBV core protein that potently inhibit HBV nucleocapsid assembly. With recombinant adenoviruses, I provided proof of principle that these HBV dominant negative mutants potently inhibit HBV replication in hepatocytes. We also performed preclinical work in woodchucks infected with the woodchuck hepatitis virus with Genzyme Corporation in preparation for clinical trials in HBV infected patients. This work led to two patents. This work laid the foundation for gene therapy of HBV, however, the interest for recombinant adenoviruses as vectors for gene therapy faded after the death of Mr. Jesse Gelsinger at the University of Pennsylvania during a clinical trial. I also characterized the properties of HBV mutants that arise during therapy with lamivudine. This was one of the first studies addressing resistance to therapy with nucleoside analogs. Furthermore, I contributed to the cloning of the Hepatitis B X interacting protein XIP: this protein is essential for the replication of HBV. XIP subsequently was found to be an integral component of the mTOR complex critically involved in amino acid sensing and activation of mTORC1 and renamed LAMTOR5. I was the leading post-doctoral fellow for these studies.

a. **P. P. Scaglioni**, M. Melegari and J. R. Wands. Characterization of Hepatitis B Virus core mutants that inhibit viral replication. Virology. 1994. 205: 112-120. PubMed PMID: 7975206.

b. **P. P. Scaglioni**, M. Melegari, M. Takahashi, J. Roy Chowdhury, and J. R. Wands. Use of dominant negative mutants of the hepadnaviral core protein as antiviral agents. Hepatology. 1996. 24: 1010-1017. PubMed PMID: 8903368.

c. M. Melegari*, **P. P. Scaglioni***, and J. R. Wands. (*First authorship shared) Hepatitis B virus mutants associated with 3TC and famciclovir administration are replication defective. *Hepatology*. 1998. 27: 628-33. PubMed PMID: 9462667.

d. M. Melegari*, **P. P. Scaglioni***, and J. R. Wands. (*First authorship shared) Cloning and characterization of a novel hepatitis B virus x binding protein that inhibits viral replication. *Journal of Virology*. 1998. 72: 1737-43. PubMed PMID: 9499022; PubMed Central PMCID: PMC109461.

Patents:

Inhibition of Hepatitis B Replication. Patent # WO09700698A1

Inhibition of Viral Replication. Patent # WO 09809649A1

2. During my medical oncology training at Memorial-Sloan Kettering, I joined the laboratory of Pier Paolo Pandolfi as a post-doctoral fellow, and later, as an Instructor in Medicine. I studied the properties of the Promyelocytic tumor suppressor (PML), a component of the PML-RARA oncoprotein of acute promyelocytic leukemia (APL). I found that PML has a tumor suppressive role in several solid malignancies, a novel concept at the time since it was thought that PML was a myeloid tumor suppressor. I found that PML is frequently lost in a wide variety of tumors through aberrant ubiquitination triggered a direct phosphorylation of a PML degron by the CK2 protein kinase. Importantly, pharmacologic inhibition of CK2 restores PML and its tumor suppressor function in cancer cells. Furthermore, I determined that PML loss accelerates mutant KRAS tumorigenesis in a mouse model of lung cancer, providing genetic evidence that PML is a tumor suppressor in vivo. This work was important because it highlighted the fact that tumor suppressors can be lost through ubiquitin mediated degradation, an emerging oncogenic mechanism at the time, which can be targeted therapeutically. I was the leading post-doctoral fellow for these studies.

a. R. Bernardi, **P. P. Scaglioni**, S. Bergmann, H.F. Horn, K.H. Vousden and P. P. Pandolfi. PML regulates p53 stability by sequestering Mdm2 to the nucleolus. *Nat. Cell. Biol.* 2004. 6:665-672. PMID: 15195100.

b. L.C. Trotman, A. Alimonti, **P.P. Scaglioni**, J.A. Koutcher, C. Cordon-Cardo, P.P. Pandolfi. Identification of a tumour suppressor network opposing nuclear Akt function. *Nature*. 2006. 25:523-527. PubMed PMID: 16680151; PubMed Central PMCID: PMC1976603.

c. **P.P. Scaglioni**, T. Yung, L. F. Cai, H. Erdjument-Bromage, P. Tempst, J. Teruya-Feldstein, P. P. Pandolfi. A CK2-dependent pathway for PML degradation upon cellular and oncogenic stress. *Cell*. 2006. 126:269-83. PMID: 16873060.

d. T.H. Shen, H.K. Lin, **P.P. Scaglioni**, T.M. Yung, P.P. Pandolfi. The Mechanisms of PML-Nuclear Body Formation. *Mol Cell*. 2006. 24:331-9. PubMed PMID: 17081985; PubMed Central PMCID: PMC1978182.

3. As an independent investigator, I also pursued studies aimed at the identification of vulnerabilities of lung cancer cells that express mutant KRAS, an oncogene considered undruggable. With an approach that integrates mouse lung cancer models, studies in cancer derived cell lines and with synthetic lethal screenings we identified several vulnerabilities of lung cancer. We found that the PI3K/mTOR signaling pathway is required for the proliferation of lung cancer cells and that dual inhibition of PI3K and mTOR leads to potent radiosensitizing effects. We determined that Focal adhesion kinase is required for the viability of mutant KRAS lung cancer: these findings led to the execution of a multicenter phase II clinical trial with a focal adhesion kinase inhibitor defactinib in lung cancer patients (ClinicalTrials.gov Identifier: NCT01951690). This work is relevant because it identified novel therapeutic targets. Finally, we determined several vulnerabilities of mutant KRAS lung cancer, providing evidence for novel therapeutic approaches for this devastating cancer. I was the PI or co-PI of these studies.

a. G. Konstantinidou, G. Ramadori, F. Torti, K. Kangasniemi, R.E. Ramirez, Y. Cai, C. Berhens, M.T. Dellinger, I.I. Wistuba, R.A. Brekken, J. Teruya-Feldstein and **P.P. Scaglioni**. RHOA-FAK signaling is required for the maintenance of K-RAS-driven adenocarcinomas. *Cancer Discovery*, 2013. 3:444-57. Article **featured in World Biomedical Frontiers**. PubMed PMID: 23358651; PubMed Central PMCID: PMC3625467.

b. Tang K.J., Constanzo, J., N. Venkateswaran, M. Melegari, M. Ilcheva, J.C. Morales, F. Skoulidis, J.V. Heymach, D.A. Boothman, **P.P. Scaglioni**. Focal Adhesion Kinase regulates the DNA damage response and its inhibition radiosensitizes mutant KRAS lung cancer. *Clin Cancer Res*. 2016. pii: clincanres.2603.2015. [Epub ahead of print]. PMID: 27220963

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4. As an independent investigator, I pursued studies to identify the contribution of PML to APL and to lung tumorigenesis. Both PML and PML-RARA are SUMOylated, however the functional significance of this modification is not well understood. We found that PIAS1 is the SUMO E3 ligase for both PML and PML-RARA. PIAS1 promotes the degradation of both PML and PML-RARA. We also found that PIAS1 is a novel positive regulator of MYC, playing a critical role in promoting MYC-driven lymphomagenesis. Furthermore, *Pias1* null mouse embryos have down regulation of MYC, severe impairment of yolk sac development and erythropoiesis. Thus, PIAS1 is a novel oncogenic factor. We also found that arsenic trioxide, a drug of choice for the treatment of Acute promyelocytic leukemia, activates PIAS1 leading to PML-RARA SUMOylation and subsequent ubiquitin mediated degradation. Thus, PIAS1 is critical for the therapeutic action of arsenic trioxide, a drug of choice in the treatment of APL. These studies highlight the role of PIAS1 and of the SUMOylation machinery in oncogenesis and in determining the response to targeted therapy. I was the PI of these studies.

a. A. Rabellino, B. Carter, G. Konstantinidou, S.Y. Wu, A. Rimessi, L. A. Byers, J.V. Heymach, Luc Girard, C.M Chiang, J. Teruya-Feldstein and P. P. Scaglioni. The SUMO E3-ligase PIAS1 regulates the tumor suppressor PML and its oncogenic counterpart PML-RARA. *Cancer Research*, 2012. 72:2275-84. PubMed PMID: 22406621; PubMed Central PMCID: PMC3342450.

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Complete List of Published Work in SciENCv

<http://www.ncbi.nlm.nih.gov/myncbi/pier%20paolo.scaglioni.1/cv/129751/>

D. Research Support

Ongoing Research Support

CPRIT RP140672

Individual Investigator Research Award

8/31/2014-8/30/2017

Mutant KRAS reprograms lipid metabolism exposing beta-oxidation as a novel therapeutic target in lung cancer lung cancer.

Major goal: with this grant we propose to determine the role of fatty acid beta-oxidation in metabolic reprogramming of mutant KRA sung cancer cells. **Role: PI**

Texas 4000

One time gift 2016

Synthetic lethal interventions for KRAS mutant lung cancer

Major Goal: the aim of this research is to test the feasibility of inactivating genes that are synthetic lethal with by adenoviral delivery of CRISPR/CAS9 technology in the mouse lung in vivo. **Role: PI**

Grant #RP150519

6/1/2016-5/31/2020

Cancer Prevention and Research Institute of Texas (CPRIT)

Title: "Defining and Defeating Mechanistic Subtypes of KRAS-mutant Lung Cancers".

Major Goal: this research program grant proposes to identify and target vulnerabilities of mutant KRAS that occur in the contest of specific single codon KRAs mutations or in the setting of multiple co-mutations, and to better understand how to target these co-mutations. **Role: PI of project 2.**

No overlap with the other funded grants

Grant #13-068-01-TBG

1/1/2013-12/31/2017 (NCE)

American Cancer Society Scholar Award

Major Goal: Identification of critical components of the K-RAS network in lung cancer

Major Goal: with this grant we will identify vulnerabilities of mutant K-RAS lung cancer. **Role: PI**

5P50 CA70907-15 (Minna)

9/1/1998-4/30/2019

NIH/NCI

University of Texas SPORE (Special Program of Research Excellence) in Lung Cancer

Major Goal: translation of findings to and from the laboratory and the clinic to result in improvement in the diagnosis, treatment, and prevention of lung cancer. It is a joint effort of UTSW and MDACC.

Role: Co-PI of Project #3 "Preclinical Development and Clinical Testing of MEK and PI3K Targeted Therapy for KRAS-mutant NSCLC as a Method of Radiosensitization and Metastasis Inhibition"

Grant #RP101251 (Magensdorf)

Cancer Prevention and Research Institute of Texas (CPRIT)

9/1/2012-8/31/2017

Development of Nuclear Receptor and Coregulator Profiles for Diagnostic and Therapeutic for diagnostic and therapeutic (theragnostic) targeting of breast and lung cancers.

Major goal: this grant proposes to determine whether nuclear receptors and nuclear co-receptors can be used as risk stratifiers or therapeutic targets in lung and breast cancers. **Role: collaborator**

Completed Research Support

Grant # LC110229

9/1/2012-8/31/2016 (NCE)

CDMRP LCRP Investigator-Initiated Research Award

Deconstruction of oncogenic K-RAS signaling reveals focal adhesion kinase as a novel therapeutic target in NSCLC

Major Goal: with this grant we will characterize the role of FAK signaling in mutant K-RAS induced NSCLC tumorigenesis. **Role: PI**

RO1 CA 137195A1

7/1/2009-6/30/2015 (NCE)

NIH/NCI

Characterization and Drug Targeting of the PML Tumor Suppressor in Lung Cancer.

Major Goal: this grant proposes studies to identify the mechanisms that mediate PML tumor suppressive function with an approach that integrates a biochemical and molecular approach with studies in mouse models of lung cancer. **Role: PI**

Molecular Cancer Research



Nullifying the *CDKN2AB* Locus Promotes Mutant K-ras Lung Tumorigenesis

Katja Schuster, Niranjana Venkateswaran, Andrea Rabellino, et al.

Mol Cancer Res 2014;12:912-923. Published OnlineFirst March 11, 2014.

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Nullifying the *CDKN2AB* Locus Promotes Mutant K-ras Lung Tumorigenesis

Katja Schuster^{1,2}, Niranjan Venkateswaran^{1,2}, Andrea Rabellino^{1,2}, Luc Girard^{3,4}, Samuel Peña-Llopis^{1,2,5}, and Pier Paolo Scaglioni^{1,2}

Abstract

Lung cancer commonly displays a number of recurrent genetic abnormalities, and about 30% of lung adenocarcinomas carry activating mutations in the *Kras* gene, often concomitantly with inactivation of tumor suppressor genes *p16^{INK4A}* and *p14^{ARF}* of the *CDKN2AB* locus. However, little is known regarding the function of *p15^{INK4B}* translated from the same locus. To determine the frequency of *CDKN2AB* loss in human mutant *KRAS* lung cancer, The Cancer Genome Atlas (TCGA) database was interrogated. Two-hit inactivation of *CDKN2A* and *CDKN2B* occurs frequently in patients with mutant *KRAS* lung adenocarcinoma. Moreover, *p15^{INK4B}* loss occurs in the presence of biallelic inactivation of *p16^{INK4A}* and *p14^{ARF}*, suggesting that *p15^{INK4B}* loss confers a selective advantage to mutant *KRAS* lung cancers that are *p16^{INK4A}* and *p14^{ARF}* deficient. To determine the significance of *CDKN2AB* loss *in vivo*, genetically engineered lung cancer mouse models that express mutant *Kras* in the respiratory epithelium were utilized. Importantly, complete loss of *CDKN2AB* strikingly accelerated mutant *Kras*-driven lung tumorigenesis, leading to loss of differentiation, increased metastatic disease, and decreased overall survival. Primary mutant *Kras* lung epithelial cells lacking *Cdkn2ab* had increased clonogenic potential. Furthermore, comparative analysis of mutant *Kras*; *Cdkn2a* null with *Kras*; *Cdkn2ab* null mice and experiments with mutant *KRAS*; *CDKN2AB*-deficient human lung cancer cells indicated that *p15^{INK4B}* is a critical tumor suppressor. Thus, the loss of *CDKN2AB* is of biologic significance in mutant *KRAS* lung tumorigenesis by fostering cellular proliferation, cancer cell differentiation, and metastatic behavior.

Implications: These findings indicate that mutant *Kras*; *Cdkn2ab* null mice provide a platform for accurately modeling aggressive lung adenocarcinoma and testing therapeutic modalities. *Mol Cancer Res*; 12(6): 912–23. ©2014 AACR.

Introduction

Lung adenocarcinoma, a leading source of cancer-related mortality worldwide (1), harbors recurrent genomic abnormalities that provide a framework for the selection of targeted therapeutic agents (2–4). For these reasons, intense research efforts are under way to characterize the biologic and therapeutic significance of recurrent genomic abnormalities in non-small cell lung cancer (NSCLC). Activating mutations of *KRAS* occur in approximately 30% of lung adenocarcinomas. *KRAS* is a small GTPase that regulates several

oncogenic networks (5). Mutant *KRAS* plays a causative role in lung tumorigenesis; however, it is not sufficient for the induction of high-grade lung adenocarcinomas in the absence of cooperating mutations that often involve the *CDKN2AB* locus (6–9).

The *CDKN2AB* locus encodes for several tumor suppressors. *CDKN2A* contains *p16^{INK4A}* and *p14^{ARF}* (*p19^{Arf}* in mice), while *CDKN2B* contains *p15^{INK4B}*. Both *p16^{INK4A}* and *p15^{INK4B}* promote G₁ cell-cycle arrest by inhibiting the CDK4/CDK6 retinoblastoma family of tumor suppressors, whereas *p14^{ARF}* upregulates TP53 by inactivating its negative regulator MDM2 (10, 11). Both *p16^{INK4A}* and *p15^{INK4B}* are highly similar and appear to have originated from gene duplication (12–14), whereas *p14^{ARF}* gene expression is initiated from an exon intercalated between *p16^{INK4A}* and *p15^{INK4B}* and an alternative reading frame of exon 2 and 3 of *p16^{INK4A}* (refs. 14, 15; Fig. 1A). Several studies established that *p16^{INK4A}* and *p14^{ARF}* are bona fide tumor suppressors *in vivo*, including in mutant *KRAS* lung adenocarcinomas (6, 9, 15–17).

Despite convincing biochemical evidence that *p15^{INK4B}* is part of the TGF- β signaling pathway, much less is known regarding its tumor suppressor function *in vivo* (18). For

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (<http://mcr.aacrjournals.org/>).

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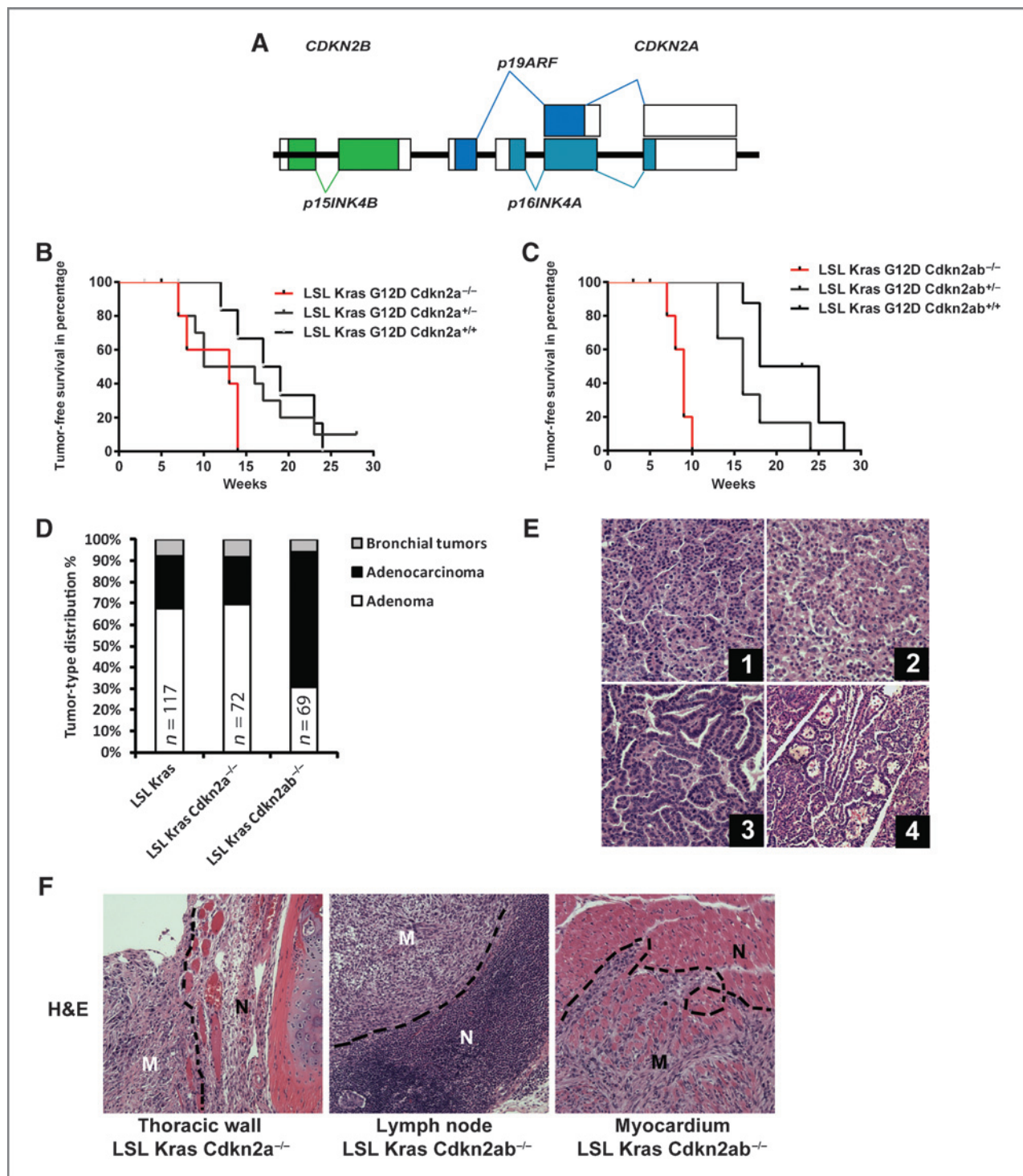


Figure 1. Loss of *Cdkn2ab* results in aggressive lung cancer in an oncogenic *Kras* conditional mouse. A, schematic of the *Cdkn2ab* locus in the mouse (not to scale). B, Kaplan-Meier survival curve of mice with the indicated genotypes ($n = 8$; $P = 0.024$, log-rank, Mantel-Cox, wild-type versus *Cdkn2a* null). C, Kaplan-Meier survival curve of mice with the indicated genotypes. Note that while LSL-Kras;*Cdkn2ab*^{+/-} mice have a half-life of 21.5 weeks ($n = 8$), LSL-Kras;*Cdkn2ab*^{-/-} mice ($n = 7$) have their half-life reduced to 9 weeks ($P < 0.0001$, log-rank, Mantel-Cox). D, Histogram of tumor type distribution of conditional mice with the indicated genotypes. Numbers of tumors counted are indicated. E, representative images of lung cancers stained with hematoxylin and eosin (H&E). (1) Lung adenoma of LSL-Kras and (2) of LSL-Kras;*Cdkn2a*^{-/-} mice. (3) Adenocarcinoma of LSL-Kras;*Cdkn2ab*^{-/-} mice and (4) bronchial tumor of LSL-Kras;*Cdkn2ab*^{-/-}, magnification 200 fold. F, metastases in the indicated genotypes and anatomic location. M, metastatic tissue; N, normal tissue. H&E staining, magnification $\times 100$.

example, *p15^{INK4B}* is rarely mutated independently of the other *CDKN2A* genes (14). Furthermore, in the absence of other mutations, *p15^{INK4B}* null mice have only a mild tumor predisposition (19). However, Krimpenfort and colleagues demonstrated that *Cdkn2ab* null mice are tumor-prone and develop an expanded tumor spectrum as compared with *Cdkn2a* null mice (20). This study led to the conclusion that *p15^{INK4B}* provides a tumor-suppressive function that is critical in the absence of *p16^{INK4A}* and *p19^{ARF}* (20). It is unknown whether *p15^{INK4B}* has a tumor-suppressive role in other tumor models or whether its status influences tumorigenesis driven by activating mutations of proto-oncogenes commonly occurring in human cancer. For instance, even though *p15^{INK4B}* loss has been reported to occur in lung adenocarcinoma, its biologic significance has yet to be established in this context (2, 21, 22).

In this article, we study the significance of *Cdkn2ab* deficiency in the biology of mutant *Kras* lung adenocarcinoma with two genetically engineered mouse models that express mutant *KRAS* in the respiratory epithelium and human lung cancer cell lines. Furthermore, we determined the mutation frequency and pattern of the *CDKN2A* and *CDKN2B* loci by analyzing The Tumor Cancer Genome Atlas database. Our data indicate that *p15^{INK4B}* provides a tumor-suppressive function in mutant *KRAS* lung tumorigenesis.

Materials and Methods

Plasmids and lentiviral particle production

The cDNA of murine *Cdkn2b* (clone ID 3495097) was obtained from Open Biosystems (Thermo Scientific) and cloned into pLVX-tight-puro (Clontech Laboratories). Recombinant lentiviral particles were generated in 293T cells according to manufacturer's procedures.

Mouse models and tumor burden assessment

Tet-op-*Kras*;CCSP-rtTA were obtained from H.E. Varmus (6), *HIST1H2Bj-GFP* mice were from the Jackson Laboratory (23), *Ink4ab^{-/-}* mice (hereafter named *Cdkn2ab^{-/-}*) were provided by Dr. Anton Berns (The Netherlands Cancer Institute, Amsterdam, the Netherlands), *LSL-Kras^{G12D}* mice were from the National Cancer Institute (NCI) mouse repository (24) and *Ink4a/Arf^{fllox/fllox}* mice were from the Jackson Laboratory (25). Mice were maintained in a mixed background (FVB/N/CD-1). Experiments were performed with F3 generation progeny or later progenies, and comparisons were made with littermates. We obtained lung-specific *KRAS* expression at 4 weeks of age either by feeding mice with doxycycline-implemented food pellets (Harlan Laboratories) or by intratracheal administration of Adenovirus-Cre at 8 weeks of age (University of Iowa, Gene transfer Vector Core; refs. 6, 26). We administered doxycycline at 4 weeks of age to attempt to develop lung tumors before the development of tumors involving other organs, a common occurrence in *Cdkn2ab* null mice. All animal studies were completed according to the policies of the University of Texas Southwestern Institutional Animal Care and Use Committee. We used digital quantifica-

tion of the area occupied by tumors to the area of total lung using the NIH ImageJ (v1.42q) software.

Primary cultures and colony formation assays

Single-cell suspensions of primary respiratory cells were sorted with a MoFlo cell sorter to select and plate GFP fluorescent cells on either irradiated or mitomycin C-treated mouse embryonic fibroblasts (MEF).

Cell lines

Human NSCLC cell lines H460 and A549 were from the Hamon Center cell line repository (UT Southwestern Medical Center, Dallas, TX).

Quantitative real-time PCR

We extracted RNA using the RNeasy Kit (Qiagen) and generated cDNA using iScript cDNA kit (Bio-Rad). For quantitative real-time PCR (qRT-PCR), we used iTaq SYBR green supermix with ROX (Bio-Rad).

Western blotting

We used the following antibodies: anti-PARP (Cell Signaling Technology), anti-HSP90 (BD Biosciences), anti-p15 (Sigma), and anti-phospho-H3 (Millipore).

Immunohistochemistry

We used the following antibodies: GFP (Chemicon), phospho-S6 ribosomal protein, HMG2A and phospho-Erk (Cell Signaling Technology), ALDH1A1 (Abcam), phospho-H3 (Millipore), NKX2-1 (Santa Cruz Biotechnology), SP-C (Chemicon), and CCSP (Santa Cruz Biotechnology). An HMG2A staining scoring system was used as follows: tumors were considered negative if no stain was visible, positive in few cells, positive in clusters of cells, and positive if >50% of tumor cells scored HMG2a positive.

Analyses of the TCGA database

Information regarding mutation, segmented copy number, methylation, and clinical data of primary lung adenocarcinomas were obtained from TCGA data portal (<https://tcga-data.nci.nih.gov/tcga>) on June 8, 2013. Only nonsilent mutations were considered as described (27). Kaplan–Meier was used to estimate the survival curves and comparisons were performed using the log-rank test using SPSS Statistics 17.0. One-way ANOVA followed by Tukey *post hoc* test was used to compare the pathologic stage among groups.

Copy number and microarray analysis of lung cancer cell lines

Most of the *KRAS* and *CDKN2A* mutations data were obtained from the COSMIC database (Sanger Institute, United Kingdom), the literature, or from our unpublished whole-exome sequencing data (28). mRNA expression microarrays were performed by us with Illumina HumanWG-6 V3 as previously described (29). They were deposited at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) under the accession GSE32036. Whole-genome Single-nucleotide polymorphism (SNP) profiling was done as previously described (30).

Results

***Cdkn2ab* deficiency accelerates mutant *KRAS* lung tumorigenesis in mice**

To characterize the effect of deficiency of genes encoded by *Cdkn2ab* on lung tumorigenesis (Fig. 1A), we generated compound mutant mice expressing mutant *KRAS* in the respiratory epithelium. We crossed *LSL-Kras^{G12D}* mice (*LSL-Kras* mice), which encode a latent mutant *Kras* gene, with mice harboring conditional alleles of *Cdkn2a* or *Cdk2ab* (20, 24, 25). We achieved lung-specific tumor development by intratracheal delivery of adenovirus encoding for the Cre recombinase.

The overall median survival of *LSL-Kras;Cdkn2a^{-/-}* was significantly decreased as compared with their littermates with a wild-type *Cdkn2a* locus, whereas *LSL-Kras;Cdkn2a^{+/-}* had a trend toward an intermediate survival between *Cdkn2a* wild-type and null genotypes (Fig. 1B). The survival of *LSL-Kras;Cdkn2ab^{-/-}* mice was significantly decreased as compared with *LSL-Kras*, *LSL-Kras;Cdkn2ab^{+/-}* or *LSL-Kras;Cdkn2a^{-/-}* mice (Fig. 1C). These findings demonstrate that complete loss of *Cdkn2ab* significantly promotes mutant *KRAS* lung tumorigenesis.

Mutant *KRAS* induces high-grade and metastatic lung adenocarcinomas in *Cdkn2ab* null mice

At the time of death, the majority of lung parenchyma was occupied by lung tumors in every genotype (Supplementary Fig. S1A–S1C). This finding suggests that death is caused by respiratory compromise.

We determined genotype-specific histologic features: lungs of *LSL-Kras* and *LSL-Kras;Cdkn2a^{-/-}* mice contained predominantly lung adenomas (Fig. 1D and E, panel 1 and 2) and occasional carcinomas with nuclear atypia (not shown). In contrast, *Cdkn2ab* null mice carried primarily high-grade lung adenocarcinomas consisting of atypical nuclei arranged in papillary structures (Fig. 1E, panel 3). Moreover, we detected tumors arising from the bronchial epithelia in all three genotypes (Fig. 1D and E, panel 4).

We observed that 44% of *LSL-Kras;Cdkn2a^{-/-}* mice carried metastatic lesions in mediastinal lymph nodes, or the thoracic wall (Fig. 1F; Supplementary Table S1). This metastatic behavior was even more pronounced in *LSL-Kras;Cdkn2ab^{-/-}* mice, where 89% of the mice carried lung tumors that metastasized to mediastinal lymph nodes and the myocardium (Fig. 1F; Supplementary Table S1). These findings indicate that *p15^{Ink4b}* deficiency leads to high-grade lung adenocarcinomas with aggressive behavior, including the property to generate locoregional metastasis.

***Kras;Cdkn2ab* null lung tumors display upregulation of proliferative markers**

We determined the level of activity of targets of *KRAS*, which have been implicated in tumorigenesis, and of the mitotic marker phospho-Histone 3 (p-H3). *LSL-Kras;Cdkn2a* null and *LSL-Kras;Cdkn2ab* null tumors were diffusely positive for p-S6 and p-Erk1/2, with more intense staining in tumor segments with atypical nuclei and papillary

morphology, whereas *LSL-Kras;Cdkn2ab^{+/-}* lung tumors were faintly positive for p-S6 and p-Erk1/2 (Fig. 2A).

The staining pattern of p-H3 was genotype dependent: *LSL-Kras;Cdkn2a^{-/-}* tumors stained positive for p-H3 mostly at the tumor edge. In contrast, *LSL-Kras;Cdkn2ab^{-/-}* tumors stained positive throughout the tumor mass. Furthermore, the number of mitotic cancer cells increases significantly in *LSL-Kras;Cdkn2ab^{-/-}* tumors as compared with *Cdkn2a^{-/-}* and *Cdkn2ab^{+/-}* lung tumors (Fig. 2A and B). We conclude that the loss of *Cdkn2ab* promotes cellular proliferation and progression of *KRAS*-driven lung tumors.

***Cdkn2ab* deficiency leads to reduction of differentiation of *KRAS^{G12D}*-driven lung tumors**

To further characterize the lung tumors that develop in *LSL-Kras;Cdkn2ab* null mice, we evaluated the lung epithelial marker NKX2.1 homeobox 1 (NKX2-1) and chromosomal high motility group protein HMGA2, which define the status of differentiation of lung adenocarcinoma driven by mutant *KRAS* in the mouse (31, 32). NKX2-1 is a master regulator of lung differentiation, which is expressed in the majority of human lung adenocarcinomas (33). Human lung tumors that are negative for NKX2-1 are poorly differentiated, display high-grade histologic features, and an aggressive behavior (33–35). NKX2-1 restrains lung cancer progression by enforcing a lung epithelial differentiation program by repressing the chromatin regulator HMGA2 (31, 32).

We found that lung tumors of *LSL-Kras;Cdkn2ab^{+/-}* and *LSL-Kras;Cdkn2a^{-/-}* mice are predominately NKX2-1-positive and HMGA2-negative (Fig. 2C and D and Supplementary Fig. S2A). In contrast, about 21% of *LSL-Kras;Cdkn2ab^{-/-}* tumors lose NKX2-1 completely (Supplementary Fig. S2A). In addition, about 73% of *LSL-Kras;Cdkn2ab^{-/-}* tumors increase their number of HMGA2-positive cells (Fig. 2C–E and Supplementary Fig. S2B). Finally, we also determined that the metastases that developed in *Cdkn2a^{-/-}* and in *Cdkn2ab^{-/-}* lung tumors stain positive for HMGA2 (Supplementary Fig. S2C). This observation strongly suggests that HMGA2-positive lung tumors have metastatic potential.

These data indicate that complete loss of the *Cdkn2ab* locus significantly promotes lung cancer tumor progression in the mouse. These observations imply that *p15^{Ink4B}* provides an important tumor suppressor function in opposing mutant *KRAS* tumorigenesis.

Transgenic mouse model of genetically labeled cancer-initiating cells

To characterize in better detail the biologic consequence of *Cdkn2ab* deficiency in oncogenic *Kras* lung adenocarcinoma, we generated mice that allow the tagging and isolation of lung cancer cells. We took advantage of *CCSP-rtTA;Tet-op-Kras* mice, which carry a transgene encoding *Kras^{G12D}* under the control of the tetracycline operator (*Tet-op-Kras*), and a transgene expressing the reverse tetracycline transactivator in the respiratory epithelium under the control of the Clara cell secretory protein promoter (*CCSP-rtTA*). *Tet-op-Kras;CCSP-rtTA* mice develop lung cancer with

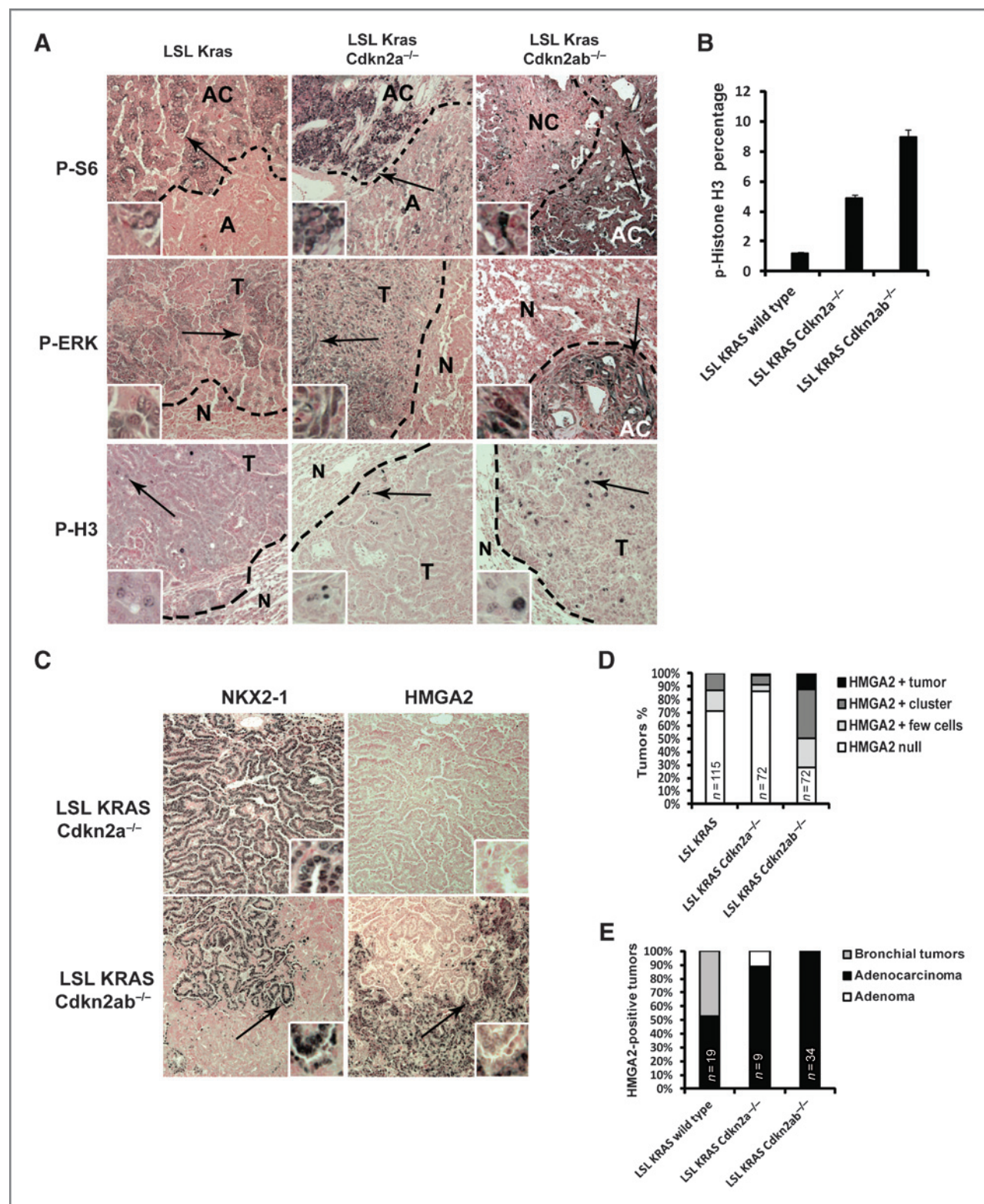
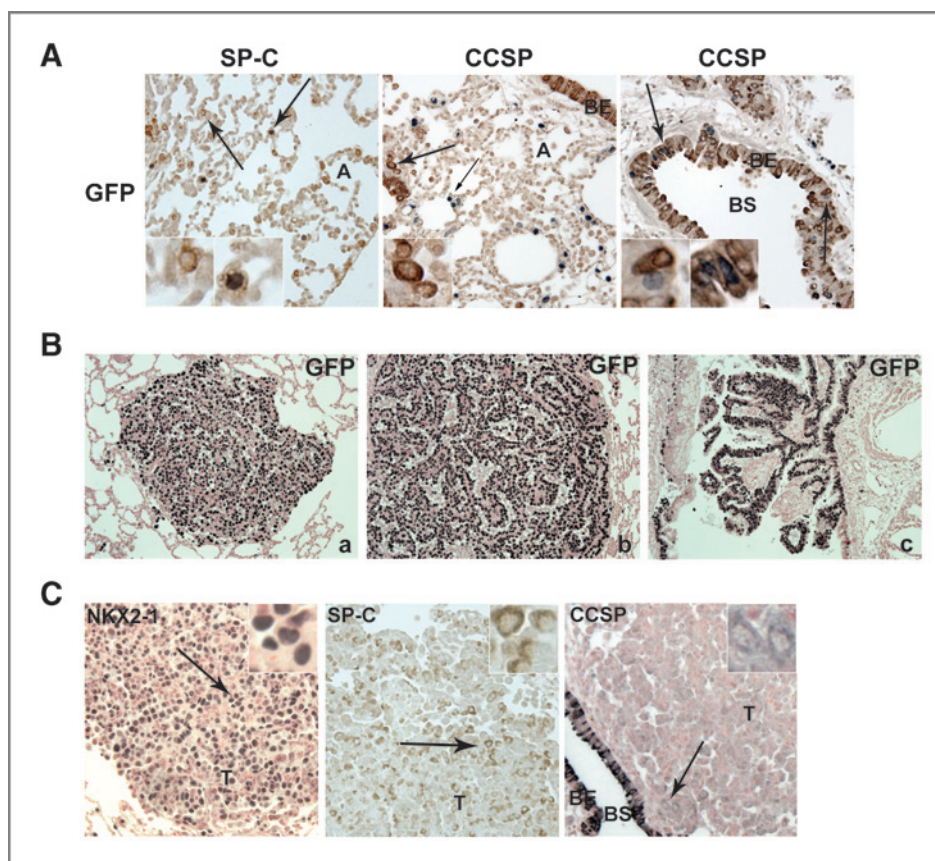


Figure 2. Loss of *Cdkn2ab* increases proliferation, and tumor progression in mutant *Kras* lung tumors. A, images of lung tumors stained with the indicated antibodies ($\times 100$). Antibody staining is shown in brown, nuclear fast red counter-stain is pink-red. Arrows show location of inlets. A, adenoma; AC, adenocarcinoma; T, tumor; N, normal tissue. B, histogram of the percentage of p-H3 positive cells lung tumors. C, Sections of lung tumors stained as indicated ($\times 100$). Arrows indicate the location of inlets. D, histogram representing percentage of lung tumors with the indicated HMGA2 staining pattern (refer to methods for scoring scale). E, anatomic location and classification of HMGA2-positive tumors. Number of tumors is indicated.

Figure 3. Lung epithelial cells and tumors genetically labeled by H2B-GFP. A, lung sections stained with the indicated antibodies. H2B-GFP-positive cells in the alveoli stain positive for SP-C, suggesting that they are alveolar type II pneumocytes. H2B-GFP staining: black gray, SP-C and CCSP staining: brown. Arrows indicated the location of the inlets. Magnification, $\times 200$. A, B, immunohistochemistry of lung tumors from *Kras-GFP* mice stained as indicated: (a) adenoma, (b) adenocarcinoma, (c) bronchial tumor ($\times 100$). C, *Kras-GFP* tumors stained as indicated. Arrows indicate the location of the inlets. Images are enlarged 200-fold. CCSP-rtTA transgene is expressed, albeit at low level, in lung cancer cells. These data independently confirm the observations of Fisher et al. regarding the pattern of expression of CCSP-rtTA (6).



100% penetrance when continuously exposed to doxycycline (6).

To visualize mutant *KRAS*-expressing lung epithelial cells, we crossed the *Tet-op-Kras;CCSP-rtTA* strain with mice that carry a transgene encoding histone *H2B* fused to *GFP* (*H2B-GFP*) under the control of the tetracycline operator (*tetO-HIST1H2BJ-GFP*; ref. 23). We determined that upon exposure to food pellets impregnated with doxycycline, *Tet-op-Kras;CCSP-rtTA;tetO-HIST1H2BJ-GFP* (*Kras-GFP* mice) transgenic mice express *H2B-GFP* in lung epithelial cells (Supplementary Fig. S3A–S3C) with the characteristics of type II pneumocytes (Fig. 3A).

When exposed continuously to doxycycline, *Kras-GFP* mice develop mutant *KRAS* lung tumors that are also positive for H2B-GFP (Fig. 3B). Lung tumor cells showed predominantly nuclear NKX2-1 staining and cytoplasmic SP-C staining (Fig. 3C). Tumor cells were predominantly negative for CCSP but we detected faint CCSP staining in a portion of tumor cells as compared with the strong staining in the bronchiolar epithelial lining (Fig. 3C). We conclude that this mouse model allows successful H2B-GFP tagging of lung cancer cells.

***Cdkn2ab* deficiency promotes the development of high-grade non-small cell lung carcinomas**

We determined the effect of *Cdkn2a* or *Cdkn2ab* deficiency on lung tumorigenesis in *Kras-GFP* mice. Significantly,

Kras-GFP;Cdkn2ab^{−/−} mice developed an increased number of tumors as well as an increased tumor burden 4.5 months after induction of mutant *KRAS* (Fig. 4A–C). In this mouse model, loss of *Cdkn2a* resulted in an intermediate number of tumors and tumor burden at 4.5 months (Supplementary Fig. S4A and S4B). We also observed an increase in tumor burden in *Kras-GFP;Cdkn2ab*^{−/−} mice at time of death (Supplementary Fig. S4C). These results indicate that *Cdkn2ab* loss promotes both mutant *KRAS* tumor initiation and progression.

Tumors arose within the lung parenchyma and alveoli in all three genotypes (Fig. 4D); however, in this model only *Cdkn2ab* null mice generated tumors that arose from the bronchial epithelia (Fig. 4D, panels 4 and 9 and Supplementary Fig. S4D). This observation suggests that *Cdkn2ab* loss reduces the threshold to develop oncogenic *KRAS*-induced tumors in distal bronchioles. In addition, *Cdkn2a* or *Cdkn2ab* null mice developed lung tumors characterized by nuclear atypia and papillary structures (Fig. 4D, panels 2, 3, 7 and 8; Supplementary Fig. S4D) that closely resembles aggressive human NSCLC (36, 37). Others and we described similar morphologic changes in lung adenocarcinomas of *Cdkn2a* null mice induced by the doxycycline-regulated mutant *Kras* transgene (6, 9, 38).

Lung tumors of *Cdkn2ab*-deficient mice show an increase in the number of cells stained positive for targets of activated *KRAS*, such as p-S6 ribosomal protein and p-ERK1/2 (Supplementary Fig. S4E). These findings suggest that

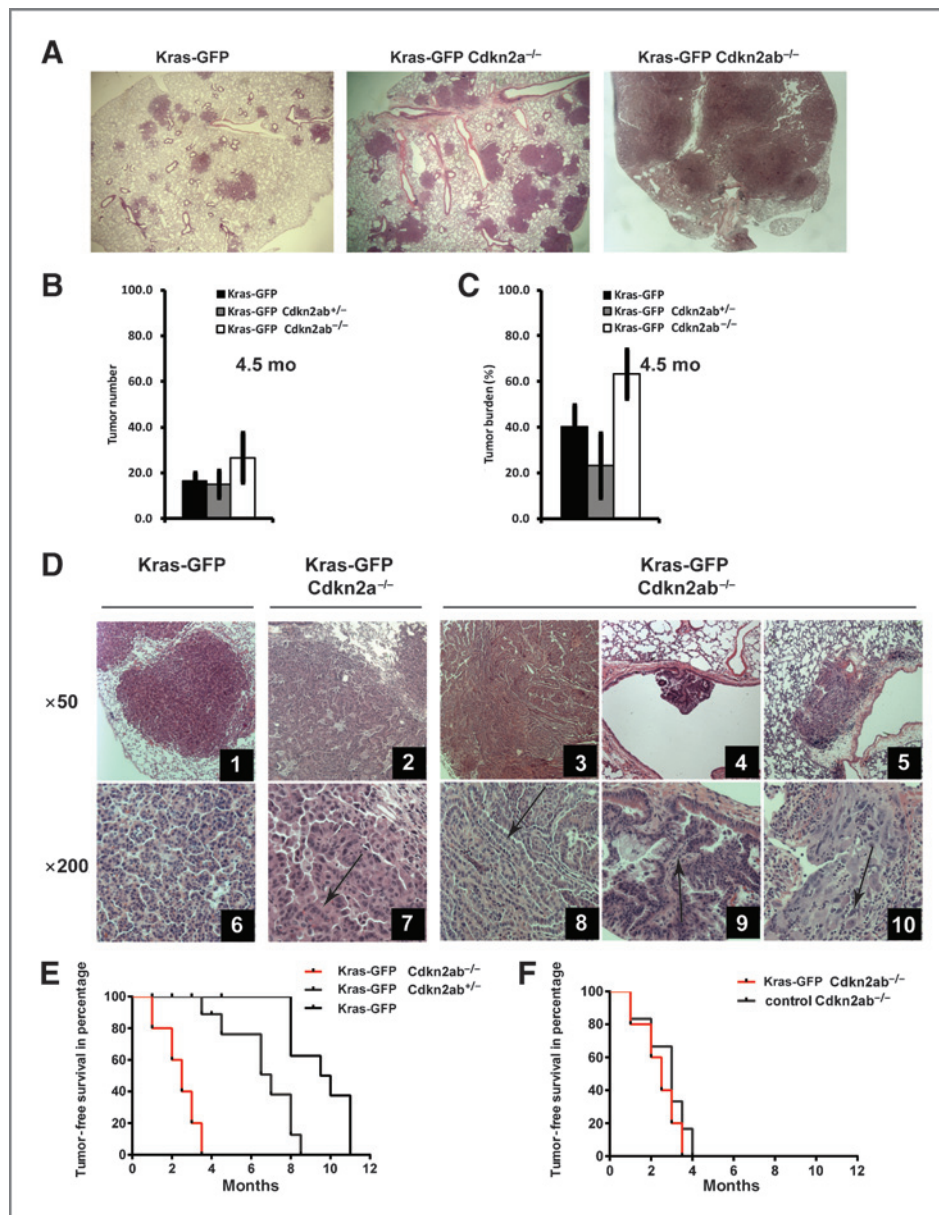


Figure 4. Loss of *Cdkn2ab* results in high-grade lung cancer and increased tumor burden. A, representative lung section of mice of the indicated genotypes 4.5 months after doxycycline exposure ($\times 15$ magnification). B and C, histograms show tumor number or burden in mice of the indicated genotype after 4.5 months of doxycycline. Note increased tumor number/burden in mutant *Kras-GFP*;*Cdkn2ab*^{-/-} mice. D, lung tumors of the indicated genotypes. Arrows indicate areas with papillary features and atypical nuclei (panels 6, 7, 8, and 9) or with poor differentiation (panel 10).

Cdkn2ab deficiency leads to increased proliferative activity. These features reproduced the characteristics of lung tumors developed in *LSL-Kras*;*Cdkn2ab*^{-/-} mice.

Kras-GFP;*Cdkn2ab*^{+/-} mice had the longest survival, *Kras-GFP*;*Cdkn2a*^{-/-} mice had an intermediate survival and *Kras-GFP*;*Cdkn2ab*^{-/-} mice had the shortest survival among the three groups (Supplementary Fig. S4G). These differences, however, were due mainly to sarcomas and lymphomas, which preclude the assessment of lung cancer-specific mortality (Supplementary Fig. S4H and Supplementary Table S2).

H2B-GFP-positive epithelial cells form colonies *in vitro*

To evaluate the properties of lung epithelial cells marked by H2B-GFP, we obtained single-cell suspensions from the

lungs of *Kras-GFP* and *CCSP-rtTA*;tetO-*HIST1H2BJ-GFP* control transgenic mice (control-GFP mice). We found that one of 85 H2B-GFP-positive oncogenic *KRAS*-expressing lung epithelial cells formed colonies, whereas only 1 of 150 GFP-positive, but wild-type *Kras* lung epithelial cells were able to do so (Supplementary Fig. S5A and S5B). We did not detect any difference in the number of lung epithelial cells present in colonies of either control or mutant *KRAS*-expressing GFP-positive cells (Supplementary Fig. S5C). This finding suggests that mutant *KRAS* promotes clonogenicity, but not cell proliferation under these conditions.

We were able to passage these cells up to 5 times onto feeder cultures, suggesting that they can be propagated *in vitro* and have short-term self-renewal abilities. Of note, similar colony formation ratios were reported for bronchoalveolar stem cells,

putative epithelial stem cells present at the bronchoalveolar junction (39).

We were unable to propagate H2B-GFP-positive cells obtained from the respiratory epithelium beyond 5 passages due to loss of viability. In contrast, H2B-GFP-positive primary lung epithelial cells obtained from *Kras-GFP; Cdkn2a*^{-/-} or *Kras-GFP; Cdkn2ab*^{-/-} mice readily give rise to cell lines when grown as monolayer cultures in conventional tissue culture plates and retain a subpopulation of H2B-GFP-positive cells for up to 20 passages (Supplementary Fig. S5D and S5E). Moreover, H2B-GFP-positive cells are positive for lung epithelial markers and the cancer stem cell marker ALDH1A1 (Supplementary Fig. S5F). As expected, only H2B-GFP-positive cells express mutant *Kras* (Supplementary Fig. S5G). Moreover, mutant *Kras* expression is doxycycline dependent and cannot be reinduced (Supplementary Fig. S5H). Taken together, these data suggest that deficiency of the genes of the *Cdkn2ab* locus promote immortalization of the respiratory epithelial cells.

Loss of *p15^{INK4B}* promotes mutant KRAS tumorigenesis in human lung cancer

To determine the frequency of the loss of genes of the *CDKN2AB* locus, we determined the mutation status of *p15^{INK4B}*, *p16^{INK4A}* and *p14^{ARF}* in a panel of 23 human NSCLC lines which express mutant KRAS. For this purpose, we used direct sequencing to detect point mutations and SNP analysis to detect gene copy number alteration (Table 1). We established that 6 of the cell lines carried homozygous deletions of *CDKN2AB* and 15 cell lines carried at least heterozygous deletion of *CDKN2AB*. Only two cell lines (HCC44 and H1155) did not lose a single copy of the *CDKN2AB* allele. We validated nullizygosity of the *CDKN2AB* locus in A549 and H460 by qRT-PCR (Fig. 5A). These findings indicate that loss-of-function of all three tumor suppressors of *CDKN2AB* is a common event in human lung adenocarcinoma.

We next analyzed patient data from The Cancer Genome Atlas (TCGA) database. Data with mutation, copy number, and methylation was available for 323 lung adenocarcinoma patients and showed that 30% ($n = 96$) had oncogenic *KRAS* mutations. In addition, 51 of the 96 patients (53%) with *KRAS* mutations also had two-hit *CDKN2A* inactivation by mutation, chromosomal deletion, and/or promoter methylation. Of these patients, 17 (18%) also showed inactivation in the *CDKN2B* locus by homozygous deletions. None of the patients in this database showed inactivation of *CDKN2B* but not of *CDKN2A*. These data indicate that the loss of the *CDKN2AB* locus is a common occurrence in human NSCLC (Fig. 5B). Notably, deficiency of *CDKN2A* is associated with higher pathologic stage (Supplementary Table S3), poorer overall survival (Fig. 5C), and progression-free survival (Fig. 5D) in patients with mutant *KRAS* lung adenocarcinoma. While there is a tendency to poorer progression-free survival with inactivation of *CDKN2B* in addition to *CDKN2A* ($P = 0.19$), this difference was not significant. Anyway, inactivation of either *CDKN2A* or *CDKN2AB* is associated with poorer progression-free sur-

Table 1. *CDKN2AB* status of *KRAS* mutant NSCLC lines

Cell line	Tumor subtype	CDKN2AB status	KRAS
A549	Adenocarcinoma	HOMO	MUT
H460	Large cell	HOMO	MUT
H1944	Adenocarcinoma	HOMO	MUT
HCC1171	NSCLC	HOMO	MUT
HCC4017	Large cell	HOMO	MUT
HOP-62	Adenocarcinoma	HOMO	MUT
H23	Adenocarcinoma	HET	MUT
H157	Squamous	HET	MUT
H358	Adenocarcinoma	HET	MUT
H441	Adenocarcinoma	HET	MUT
H1355	Adenocarcinoma	HET	MUT
H1373	Adenocarcinoma	HET	MUT
H1734	Adenocarcinoma	HET	MUT
H2009	Adenocarcinoma	HET	MUT
H2122	Adenocarcinoma	HET	MUT
H2347	Adenocarcinoma	HET	MUT
H2887	NSCLC	HET	MUT
HCC366	Adenosquamous	HET	MUT
HCC461	Adenocarcinoma	HET	MUT
HCC515	Adenocarcinoma	HET	MUT
Calu-6	Adenocarcinoma	HET	MUT
H1155	Large cell	WT	MUT
HCC44	Adenocarcinoma	WT	MUT

NOTE: *CDKN2AB* mutation status is indicated as WT, wild type; HOMO, homozygous loss; HET, heterozygous loss; MUT—*KRAS* mutation. Pathologic classification of cell lines is indicated as it was given at the time the cell lines were raised.

vival and overall survival (Supplementary Fig. S6A and S6B) independently of the *KRAS* mutation status.

p15^{INK4B} expression promotes growth arrest in human lung cancer cells

To determine the functional effect of *p15^{INK4B}* expression *in vitro*, we ectopically expressed *p15^{INK4B}* in mutant *KRAS* human lung cancer cell lines that also carry homozygous deletions of the *CDKN2AB* locus. We determined that reintroduction of *p15^{INK4B}* leads to an increase in the percentage of A549 and H460 cells in the G₁ phase of the cell cycle (Fig. 6A). Furthermore, we noticed a significant increase of the percentage of subdiploid cells in A549 cells, which indicate the presence of apoptosis. Western blot analysis confirmed that expression of *p15^{INK4B}* downregulates the phosphorylation of mitotic marker p-H3 (Ser 10; Fig. 6B). These results suggest that the expression of *p15^{INK4B}* promotes a tumor suppressor program that counteracts the proliferative effects of mutant *KRAS* in NSCLC cells.

Discussion

Recent technical advancements have allowed a systematic evaluation of statistically recurrent somatic mutations in

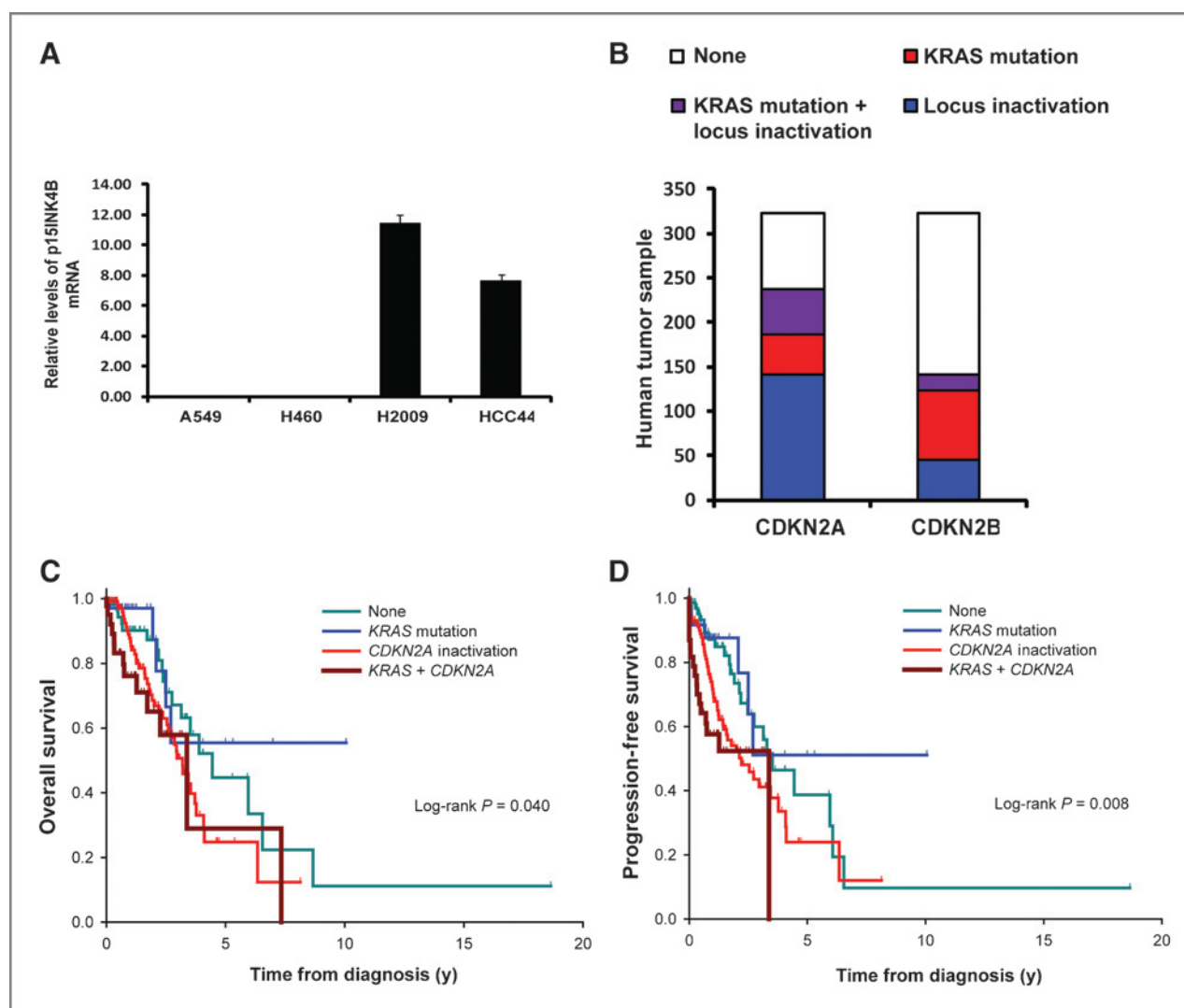


Figure 5. *CDKN2AB* status in human mutant *KRAS* lung adenocarcinoma. A, histogram of *p15^{INK4B}* mRNA in NSCLC cell lines normalized to *GAPDH*. B, histogram shows the frequency of *KRAS* mutations and *CDKN2A* or *CDKN2B* inactivation in lung adenocarcinoma in the TCGA data set. C, overall survival and D, progression-free survival curves of patients with lung adenocarcinoma carrying mutant *KRAS* and/or inactivation of *CDKN2A*.

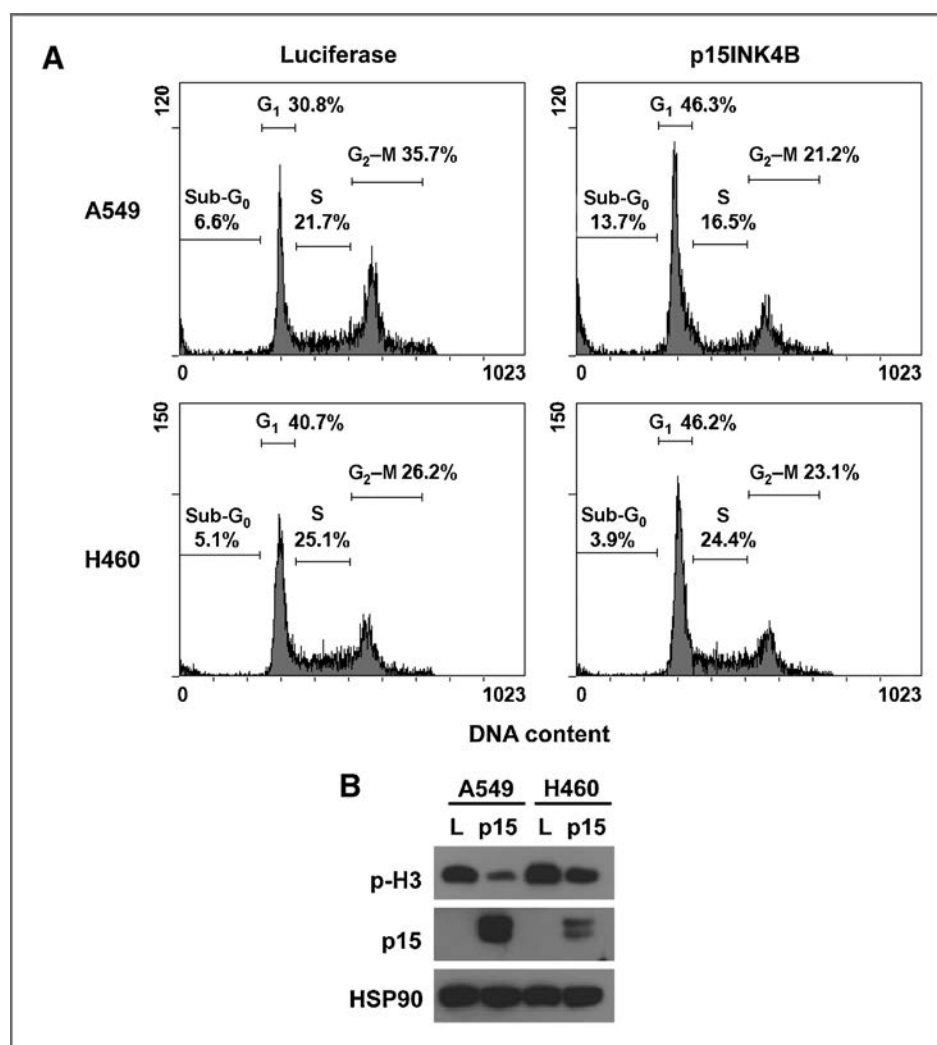
NSCLC (2, 3). This data set holds the promise to better understand lung tumorigenesis and to identify novel treatments for patients with lung cancer.

In this study, we show that genetic inactivation of the *CDKN2A* and *CDKN2B* loci is a common event in mutant *KRAS* lung adenocarcinoma. By analyzing the TCGA database, we determined that inactivation of *CDKN2A* by either somatic mutation, chromosomal deletion, and/or promoter hypermethylation, and inactivation of *CDKN2B* by homozygous deletion occur in about 50% and 20% of human lung adenocarcinomas, respectively. Previous reports did not have sufficient power to determine whether inactivation of the genes of *CDKN2AB* occurs with significant frequency in mutant *KRAS* lung cancer (2, 40). By analyzing the large and comprehensive TCGA sample cohort, it is apparent that inactivation of *CDKN2AB* occurs at a significantly higher frequency than previously noted. Furthermore, we noticed

that loss of *CDKN2B* rarely, if ever, occurs in the absence of *CDKN2A* somatic inactivation. Our analysis of the TCGA data set also indicates that loss of *CDKN2A* is associated with higher stage, higher incidence of metastasis, and consequently, decreased overall survival in patients with mutant *KRAS* lung cancer. These findings have significant clinical implications since they suggest that mutant *KRAS* lung cancers with two-hit inactivation of *CDKN2A* identify a subset of patients with high-risk disease. In this regard, it is of interest that these patients could benefit from treatment with focal adhesion kinase inhibitors (9).

We recently reported that expression of oncogenic *KRAS* in the respiratory epithelium significantly reduced the longevity of *p16^{Ink4a}/p19^{Arf}* null mice (9). We observed a further increase in lung cancer-mediated mortality in mutant *Kras* mice conditionally deficient in the entire *Cdkn2ab* locus. This observation suggests a model whereby *p15^{INK4B}* loss

Figure 6. Reintroduction of p15^{INK4B} in *CDKN2AB*-deficient lung cancer cell lines causes in growth arrest and apoptosis. **A**, cell-cycle analysis of A549 and H460 cells after 72 hours of luciferase or p15^{INK4B} expression. Both cell lines show an increase in the G₁ phase of the cell cycle. A549 cells also showed an increase in sub-G₁ fraction, suggesting that a portion of cells undergoes apoptosis. **B**, Western blot analysis of A549 and H460 cells transduced as indicated. L, luciferase.



confers a selective advantage to tumor cells that have lost p16^{INK4A} and p14^{ARF}. However, we did not detect a worse clinical outcome in patients with mutant *KRAS* lung cancers with loss of *CDKN2AB* as compared with patients with lung cancers mutant for *KRAS* and *CDKN2A*. We reason that this observation could be due to several variables, including insufficient sample size of the TCGA data set, or the presence of concurrent mutations that affect the phenotype of human cancer that are not present in genetically engineered mice.

We determined histologically that *Cdkn2a* deficiency leads to a higher percentage of high-grade lung tumors in Tet-o-*Kras* mice than in *LSL-Kras* mice, while the lung tumor phenotype of mutant *Kras*; *Cdkn2ab* null mice is consistent between the two mouse models. It is likely that these differences are either due to the strength of the promoters used to express mutant *Kras* or to the fact that we exposed mice to doxycycline at 4 weeks of age to attempt to obtain lung tumors before the emergence of tumors in other sites. We reason that *LSL-Kras* mice, owing to the fact that mutant *Kras* is expressed from the

endogenous promoter, model more closely mutant *KRAS* tumorigenesis.

Our *in vivo* cell-tracking experiments are consistent with a recent report indicating that a subset of alveolar type II cell coexpressing CCSP and SP-C give rise to lung adenocarcinomas upon mutant *KRAS* expression (41). The observation that loss of *Cdkn2ab* promotes the clonogenic ability of lung epithelial cells and their ability to readily give rise to cell lines supports the notion that *Cdkn2ab* regulates cell proliferation. This assertion is further supported by our observation that reintroduction of p15^{INK4B} induces antiproliferative effects in human NSCLC lines. We also noticed that once mutant *Kras* was extinguished in *Cdkn2ab* null lung cancer cell lines, it could no longer be induced. This observation leads to the speculation that mutant *KRAS* facilitates the maintenance of a cell state that is permissive to the activity of the CC10 promoter driving the expression of rtTA or the accessibility of the Tet-op element driving mutant *KRAS*.

Overall, our data provide new genetic evidence that the loss of the three genes residing in *CDKN2AB* promote mutant *KRAS* lung tumorigenesis by fostering cellular

proliferation, cancer cell differentiation, and metastatic behavior. We propose that mutant *Kras*;*Cdkn2ab* null mice provide a platform to accurately model aggressive mutant *Kras* lung adenocarcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Schuster, N. Venkateswaran, P.P. Scaglioni
Development of methodology: S. Peña-Llopis

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Schuster, N. Venkateswaran, S. Peña-Llopis

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Schuster, N. Venkateswaran, L. Girard, S. Peña-Llopis, P.P. Scaglioni

Writing, review, and/or revision of the manuscript: K. Schuster, N. Venkateswaran, A. Rabellino, L. Girard, S. Peña-Llopis, P.P. Scaglioni

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Schuster, N. Venkateswaran, A. Rabellino, S. Peña-Llopis
Study supervision: P.P. Scaglioni

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Focal Adhesion Kinase Regulates the DNA Damage Response and Its Inhibition Radiosensitizes Mutant *KRAS* Lung Cancer

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Abstract

Purpose: Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related deaths worldwide due to the limited availability of effective therapeutic options. For instance, there are no effective strategies for NSCLCs that harbor mutant *KRAS*, the most commonly mutated oncogene in NSCLC. Thus, our purpose was to make progress toward the generation of a novel therapeutic strategy for NSCLC.

Experimental Design: We characterized the effects of suppressing focal adhesion kinase (FAK) by RNA interference (RNAi), CRISPR/CAS9 gene editing or pharmacologic approaches in NSCLC cells and in tumor xenografts. In addition, we tested the effects of suppressing FAK in association with ionizing radiation (IR), a standard-of-care treatment modality.

Results: FAK is a critical requirement of mutant *KRAS* NSCLC cells. With functional experiments, we also found that, in mutant *KRAS* NSCLC cells, FAK inhibition resulted in persistent DNA damage and susceptibility to exposure to IR. Accordingly, administration of IR to FAK-null tumor xenografts causes a profound antitumor effect *in vivo*.

Conclusions: FAK is a novel regulator of DNA damage repair in mutant *KRAS* NSCLC and its pharmacologic inhibition leads to radiosensitizing effects that could be beneficial in cancer therapy. Our results provide a framework for the rationale clinical testing of FAK inhibitors in NSCLC patients. *Clin Cancer Res*; 22(23); 5851–63. ©2016 AACR.

Introduction

Non-small cell lung cancer (NSCLC) is associated with an aggressive course and poor prognosis. These features are due to the fact that this cancer type is often diagnosed at a stage not amenable to surgical resection. In addition, NSCLC is either endowed with or acquires resistance to available medical treatments. Accordingly, NSCLC is a leading cause of cancer-related deaths worldwide (1). In NSCLC several known oncogenes promote tumorigenesis and predict response to therapy. For instance, approximately 40% of NSCLC harbor mutations of *KRAS*, *EGFR*, *ALK* translocation, or amplification of *c-MET* (2, 3). While there

are specific inhibitors of *EGFR*, *ALK*, or *MET* that cause clinically meaningful, but short-lived antitumor responses, mutant *KRAS* remains undruggable (4, 5). Thus, therapeutic options are still limited for patients with oncogenic *KRAS* NSCLC.

KRAS belongs to a family of small guanosine triphosphatases (GTPase). Tumor-associated mutations lock *KRAS* in a constitutively active state (i.e., oncogenic *KRAS*; refs. 6, 7). When bound to GTP, *KRAS* activates several critical cell proliferation and survival signals, which include the PI3K/mTOR, MEK1/2/ERK1/2, RHOA-Focal adhesion kinase (FAK), and TBK1 signaling networks (6, 8–10).

Oncogenic *KRAS* is not only sufficient to induce lung cancer, but also required for its maintenance in transgenic mice and in human lung cancer cells (11–14). However, progression to high-grade lung adenocarcinoma requires co-occurring mutations, such as the loss of *TP53*, *CDKN2A*, *Ataxia Telangiectasia Mutated (ATM)*, or *LKB1*, which allow bypass of tumor suppressive responses induced by inappropriate proliferative stimuli, DNA damage, or energetic stress (2, 3, 8, 11–13, 15–17). The loss of tumor suppressors implicated in DNA damage repair is relevant because oncogenic *KRAS* also stimulates the production of reactive oxygen species (ROS), which promote DNA damage and genomic instability (18, 19).

Because attempts to develop direct inhibitors of oncogenic *KRAS* have been unsuccessful (4), intense efforts have been spent on targeting critical components of its downstream signaling networks in preclinical models. Several PI3K, mTOR, MEK1/2, and FAK inhibitors (FAKi) are undergoing clinical testing, but they

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Translational Relevance

There is a dearth of therapeutic options for mutant *KRAS* non-small cell lung cancer (NSCLC), a disease associated with an aggressive clinical course and resistance to therapy. We report that focal adhesion kinase (FAK) represents a vulnerability of mutant *KRAS* NSCLC. Suppression or pharmacologic inhibition of FAK causes DNA damage and radiosensitizing effects that promote the therapeutic effect of ionizing radiations both in cultured cells and in lung cancer xenografts. Several FAK inhibitors have entered clinical testing, but it is still undefined in which patient population they could be effective and whether any biomarker exists to identify patients likely to respond to therapy. Thus, our findings provide a framework for their clinical development in the context of radiotherapy, a common form of therapy used for lung cancer.

have not been approved for therapy of lung cancer (20–23). Thus, there is still an urgent need for the development of therapies that target oncogenic *KRAS* tumors.

Protein tyrosine kinase 2 (PTK2), also known as *FAK*, is a non-receptor tyrosine kinase and a major mediator of integrin signaling. Upon autophosphorylation at Tyr397, FAK interacts with SRC protein kinase family members, initiating several signaling cascades that regulate cytoskeleton remodeling, cell migration and resistance to anoikis (24). FAK is amplified or overexpressed in several cancer types, including ovarian, colon, breast, and lung cancers (25, 26). Importantly, FAK inhibition is detrimental to breast and lung cancer cells: in this context, disruption of FAK is associated with alterations in the cytoskeleton or induction of senescence and activation of DNA damage pathways, respectively (8, 25). Furthermore, in lung cancer, mutant *KRAS* is a positive regulator of FAK (8). However, the mechanisms underlying senescence induction following FAK inhibition and the functional consequences of this event in cancer cells remain unexplored.

In this study, we characterized the effects of FAK suppression in a large panel of NSCLC cells representative of frequent cancer associated mutations. We found that FAK inhibition invariably impairs the viability of mutant *KRAS* NSCLC cells. In this genetic context, suppression or inhibition of FAK was accompanied by DNA damage. In addition, we demonstrate that FAK suppression synergizes with radiotherapy both *in vivo* and *in vitro*. We propose that combination therapy with FAK inhibition and ionizing radiation (IR) may lead to important clinical benefits in the treatment of NSCLC with oncogenic *KRAS*.

Materials and Methods

Cell cultures and reagents

Human NSCLC cell lines and human bronchoalveolar cells were provided by Dr. John Minna (UT Southwestern Medical Center, Dallas, TX) and cultured as described (27, 28). A549 and H460 cells expressing vector control or LKB1 were previously described (17). All cells were *mycoplasma* free and were identified by DNA fingerprinting. Supplementary Table S1 shows the mutations that they harbor according to COSMIC: Catalogue of Somatic Mutations in Cancer (Cosmic; cancer.sanger.ac.uk). FAKi PF-562,271 and VS-4718 were obtained from Selleckchem and

Verastem, Inc., respectively (29, 30). Inhibitors were added to mid-log phase cell cultures at the indicated concentrations. All other chemicals were purchased from Sigma-Aldrich.

RNA interference

Stable *FAK* mRNA knockdown was performed with lentiviral vectors containing shRNAs against *FAK* obtained from the RNAi Consortium (TRC) following procedure described previously (8). Inducible expression of *FAK* shRNA was performed with the GEPIR vector (31). See also Supplementary Materials and Methods.

Gene editing with CRISPR/CAS9

We followed established procedures to ablate *FAK* (exon 4) using the following vectors: pCW57.1 (Addgene plasmid 50661) and pLX-sgRNA (Addgene plasmid 50662; ref. 32). We selected several single clones and assessed *FAK* editing by direct sequencing and by lack of *FAK* protein by Western blot (WB) analysis.

Cell viability and proliferation curves

Cells (0.5×10^4 to 1.5×10^4) were plated in triplicate (24-well plates) 14 to 16 hours prior to exposure to pharmacologic inhibitors. At the indicated time points or 72 hours later in cell viability assays, the cells were fixed with 10% formalin and stained with 0.1% crystal violet (8).

Immunoblotting and antibodies

WB analyses were performed as previously described (33). We used the following antibodies: FAK, Tyr397 phospho-FAK, Akt, Ser473 phospho-Akt, S6, Ser235/236 phospho-S6, Ser139 γ -H2AX (Cell Signaling Technology), H2AX (Bethyl), β -Tubulin and GAPDH (Santa Cruz Biotechnology), Vinculin and LKB1 (Cell Signaling Technology).

Colony formation assays

For colony formation assays in plastic, we plated 500 to 1,500 cells in 60-mm tissue culture dishes and scored colonies of >50 normal-appearing cells after 12 to 30 days. For soft-agar colony assays, we seeded 2,000 cells/well on semisolid agar medium in a 6-well plate in triplicate and after 14 to 21 days, colonies larger than 50 μ m were counted using an inverted microscope (34).

Flow cytometry

Cells were allowed to adhere overnight. When indicated, cells were treated with PF-562,271 and/or IR (2 Gy). Analysis of the cell cycle and percentage of cells stained with propidium iodide (PI; Sigma-Aldrich) and γ -H2AX (Millipore) were performed following a standard procedure with a FC500 Beckman Coulter flow cytometer using the WinMDI V2.8 software (35, 36).

Plasmids, transfections, and retroviral transductions

pMXs-Puro-GFP-*Fak* was obtained from Addgene (FAK-Plasmid #38194). Retroviral transductions were performed as described (37).

Expression profiling

Gene expression profiles were obtained from exponentially growing HBEC cells transduced with pMXs-Puro-GFP or pMXs-Puro-GFP-*Fak*. Microarray results have been deposited in NCBI's

Gene Expression Omnibus and are accessible through GEO series accession number GSE72470. See also Supplementary Data.

Clonogenic survival assays after exposure to IRs

Clonogenic assays were performed as described previously (38). Surviving fractions (SF) were derived using the number of colonies formed after treatment, divided by the number of cells seeded multiplied by plating efficiency. Cells were plated in triplicate onto 60-mm dishes 14 to 16 hours prior to irradiation. We added PF-562,271 at indicated concentrations to the cells four hours before irradiation. Drug-containing medium was replaced with drug-free medium after 48 hours. Cells were irradiated with a Mark I cesium irradiator (1.31 Gy/min). We scored colonies of >50 normal-appearing cells 12 to 30 days after treatment and graphed the SF versus dose of IR (Gy). Do (relative dose of IR required for 37% lethality on a log-phase kill curve), Dq (inherent DNA repair capacity: dose (Gy) required to eliminate the survival curve shoulder) and dose enhancement ratios (DERs at LD₅₀ and LD₂₀) were calculated as described (39).

Immunofluorescence

Immunofluorescence was performed as described previously using γ -H2AX (Millipore), TP53BP1 (Bethyl), and FAK (Abcam ab40794) antibodies (8).

Mouse studies

Xenograft experiments using T2.2 FAK wild-type (FAK⁺) and T2.2 FAK-null (FAK⁻) H460 NSCLC cells were performed by subcutaneous inoculation of cells into 6-week-old female athymic nude mice. Mice with xenograft tumors of 300 mm³ (7 mice/group) were treated with IR. Mice were irradiated with five 4-Gy fractions every other day for 10 days using an X-RAD 320 irradiator (Precision X-Ray, Inc.) to deliver local irradiation to the flank or thigh of lead-shielded mice. Tumor volumes were calculated every other day using the formula: (length \times width²)/2. All studies were performed according to the guidelines of the UT Southwestern Institutional Animal Care and Use Committee.

Statistical analyses

All data presented are the average \pm standard deviations of experiments repeated three or more times. Significance was determined using two-tailed unpaired Student *t* tests or one-way ANOVA. Curve fitting for the radiosensitization experiments was performed using the linear-quadratic formula which has two components of cell killing: one is proportional to the dose of IR (aD) and the other is proportional to the square of the dose of IR (bD²): exp (aD+bD²) (38, 40).

Results

Pharmacologic inhibition or silencing of FAK impairs the viability of lung cancer cells

The goal of our study was to shed light on the function of FAK in lung cancer cells and to leverage this knowledge to identify novel therapeutic opportunities. Pharmacologic inhibition of FAK with the small molecule inhibitor (FAKi) PF-562,271 led to a striking inhibition of cell viability in a panel of NSCLC cell lines (detailed genotype information is provided in Supplementary Table S1). We noted that the IC₅₀ of mutant KRAS NSCLC cell lines ranged between 2 and 4 μ mol/L, while in wild-type KRAS cell lines the

IC₅₀ was 8 μ mol/L or higher (Fig. 1A; Supplementary Table S2). When used at a concentration between 2 and 4 μ mol/L, PF-562,271 led to approximately a 50% reduction of auto-phosphorylation of FAK at Y397 (P-FAK, the active form of FAK), both in wild-type and in mutant KRAS NSCLC cells (Fig. 1B; Supplementary Fig. S1A). We obtained a similar inhibition of cell viability and P-FAK when treating NSCLC cells with VS-4718, a FAKi structurally distinct from PF-562,271 (Supplementary Fig. S1B–S1D).

Of note, pharmacokinetic studies have demonstrated that PF-562,271 and VS-4718 (formerly known as PND-1186) reach a concentration of 1 to 2 μ mol/L *in vivo* (29, 41). Notably, PF-562,271 inhibits its target, causing antitumor effects *in vivo* in a mutant KRAS lung cancer model (8).

Integrin engagement with the extracellular matrix has been implicated in the activation of FAK; thus, we tested whether culturing NSCLC cells on collagen coated plates would affect their vulnerability to FAKi. However, culturing NSCLC cells on collagen-coated plates did not affect their sensitivities to FAKi (Supplementary Fig. S1E–S1G). Taken together, our findings suggest that pharmacologic inhibition of FAK may be of therapeutic value in mutant KRAS NSCLC.

To validate, with an alternative technique, the results obtained with FAKi, we inactivated FAK genetically in a panel of 21 NSCLC cells harboring mutant KRAS or wild-type KRAS, mutant EGFR, the EML4–ALK fusion gene or MET amplification and loss/mutations of the TP53, CDKN2A/B, or LKB1 tumor suppressors (detailed genotype information is provided in Supplementary Table S1). We found that silencing FAK invariably leads to loss of viability in mutant KRAS lung cancer cells (Fig. 1C). Notably, the degree of FAK silencing was comparable between wild-type and mutant KRAS NSCLC cells (Fig. 1C–D; Supplementary Fig. S1H). In contrast, the effect of FAK silencing on cell viability was not consistent in lung cancer cells carrying genotypes other than mutant KRAS. For instance, FAK silencing strikingly reduced the viability of H1975 (mutant EGFR) and H1993 (MET amplified) cells, but not of H1650 and HCC827 (mutant EGFR) or H920 cells (MET amplified). We also noticed that the vulnerability to FAK silencing was comparable between mutant KRAS cells that carry p53, CDKN2a, or LKB1 mutations (Fig. 1C; Supplementary Table S1). This result confirms and extends our previous observations in a smaller sample size of mutant KRAS NSCLC cells, providing further support to the notion that FAK is a therapeutic target in mutant KRAS NSCLC (8).

It is well known that small molecule inhibitors of protein tyrosine kinases as well as RNAi-mediated gene silencing, may lead to off-target effects. Thus, we ablated FAK by CRISPR/CAS9 gene editing in H460-mutant KRAS lung NSCLC cells, as a representative example of the NSCLC used in our studies. We carried out our analysis in two independent FAK-null (T2.2 FAK⁻ and T2.7 FAK⁻) and wild-type FAK (T2.2 FAK⁺ and T2.7 FAK⁺) H460 clones (Fig. 1E). We determined that, also in this setting, loss of FAK dramatically affects the proliferative capacity of H460 cells (Fig. 1F and G).

FAK is required for the oncogenic properties of mutant KRAS NSCLC cells *in vitro*

Next, we determined that FAK silencing impairs the ability of mutant KRAS H460, but not of wild-type KRAS H522 NSCLC cells, which we chose as representative examples, to

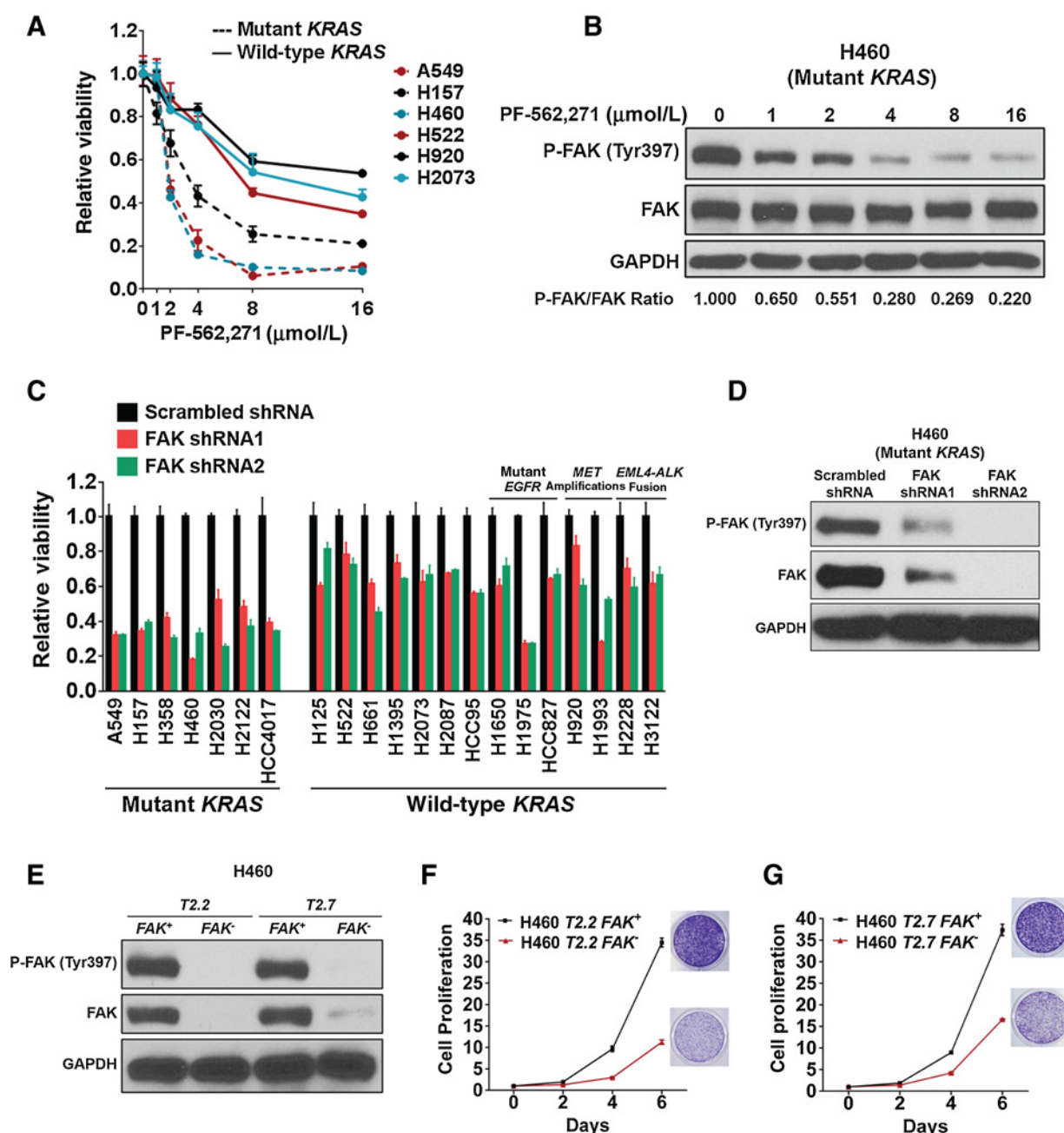


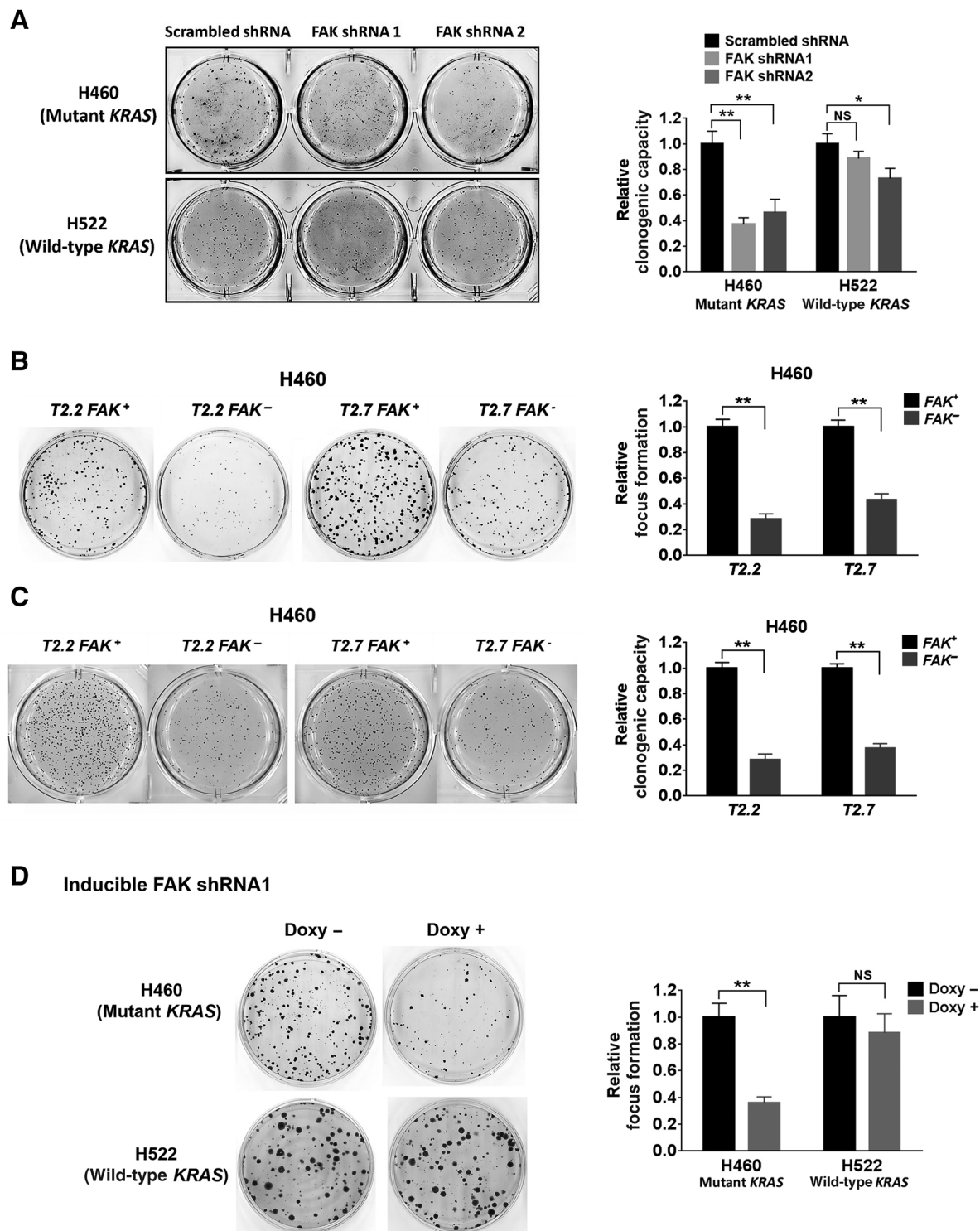
Figure 1.

Suppression of FAK leads to loss of viability in mutant *KRAS* NSCLC cells. **A**, Viability assay of NSCLC cell lines treated with FAKi PF-562,271 at the indicated concentrations. **B**, WB of H460-mutant *KRAS* NSCLC cells treated with the indicated concentrations of PF-562,271. **C**, histogram showing viability of NSCLC cells expressing the indicated shRNAs. The mutation status of the cell lines is indicated. **D**, WB of H460 cells expressing the indicated shRNAs. **E**, WB of CRISPR/CAS9-edited H460 cell clones T2.2 and T2.7; FAK⁺ and FAK⁻ indicate wild-type and null status, respectively. **F** and **G**, Proliferation assay of H460 T2.2 and T2.7 clones. FAK status is indicated. A representative picture of a tissue culture well is provided. Error bars are indicated.

grow in an anchorage-independent manner, a hallmark of oncogenic transformation and tumor aggressiveness (ref. 42; Fig. 2A). These cell lines are widely used in the literature for similar experiments. Furthermore, we determined that CRISPR/CAS9-mediated ablation of *FAK* significantly reduces the clonogenic ability of H460 cells when cultured on plastic dishes and in soft agar (Fig. 2B and C).

It is also noteworthy that the acute silencing of *FAK* with a retrovirus that expresses a doxycycline (doxy)-inducible *FAK* shRNA, which mimics the action of a pharmacologic agent, significantly impairs the clonogenic ability of H460 cells when grown on plastic (Fig. 2D).

Taken together, these data support the conclusion that *FAK* is required for the transforming ability of oncogenic *KRAS*,

**Figure 2.**

Suppression of FAK impairs the clonogenic ability of mutant *KRAS* NSCLC cells. **A**, Representative soft-agar colony formation assay of H460 and H522 cells transduced with the indicated shRNAs. The histogram shows a quantification of three experiments performed in triplicate. **B**, Representative focus assay of CRISPR/CAS9-edited H460 T2.2 and T2.7 clones. FAK status is indicated. The histogram shows a quantification of three independent experiments performed in triplicate. **C**, Representative soft-agar colony formation assay of CRISPR/CAS9-edited H460 cells. The histogram shows a quantification of three independent experiments performed in triplicate. **D**, Representative focus assay of H460 cells expressing the doxy-dependent FAK shRNA1 grown in the absence (–) or presence (+) of doxy, which was added 48 hours after plating. The histogram shows a quantification of three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; NS = nonstatistically significant.

which could be targeted for therapeutic purposes in lung cancer.

Suppression of FAK leads to a growth arrest in the G₂ phase of the cell cycle

Flow cytometry revealed that pharmacologic inhibition of FAK leads to a significant increase of the percentage of the G₂ phase of the cell cycle in mutant *KRAS* NSCLC cells, but not in wild-type *KRAS* H522 NSCLC cells (Fig. 3A and B). These data suggest that the effects of FAK suppression are mediated by a mechanism that utilizes a G₂ cell-cycle checkpoint.

Suppression of FAK leads to activation of the DNA damage response in mutant *KRAS* NSCLC cells

To characterize the effect of FAK suppression in NSCLC cells, we assessed the status of several targets of *KRAS* (6). We found that pharmacologic inhibition of FAK (FAKi) or FAK gene silencing did not affect p-AKT, p-ERK, and p-S6 levels (Supplementary Fig. S2A–S2E). This observation suggests that inhibitory effects on these signaling pathways do not cause the effect of FAKi on cell proliferation and viability.

To gain insight into the cellular networks affected by FAK, we performed gene expression analysis of primary human bronchoalveolar epithelial cells (HBEC) immortalized by expression of *hTERT* and *CDK4*. These cells have been extensively studied to determine the effect of oncogenic mutations in respiratory epithelial cells (28). In addition, using this cellular system we previously demonstrated that mutant *KRAS* activates FAK (8). Therefore, we reasoned that these cells are an experimental system to assess the cellular networks regulated by FAK. To this end, we compared the transcriptome of HBEC cells expressing pMXs-Puro-GFP or pMXs-Puro-GFP-*Fak*. Database for Annotation, Visualization and Integrated Discovery-based (DAVID) functional enrichment analysis and Gene Set Enrichment Analysis (GSEA) between experimental groups revealed a significant upregulation of genes that regulate G₂–M DNA damage checkpoint, TGF β signaling, and PKA signaling (Fig. 3C).

To address the relationship of FAK with DNA damage repair networks, we tested whether loss of FAK affects activation of γ -H2AX, an occurrence that was also previously reported to occur in breast cancer cells (25). Indeed, we found that silencing, pharmacologic inhibition, or ablation of FAK leads to upregulation of γ -H2AX in mutant *KRAS* NSCLC A549 and H460 cells but not in wild-type *KRAS* NSCLC H522 and H596 cells. Notably, there were no major differences in baseline γ -H2AX among the cell lines used for these experiments (Fig. 3D–F; Supplementary Fig. S2F). LKB1, which is lost in a significant percentage of mutant *KRAS* lung cancer, has been implicated in the regulation of the DNA damage response (43). To test whether this is the case also in lung cancer cells, we treated with FAKi PF-562,271 or IRs H460 (LKB1 mutant), A549 cells (LKB1 mutant) and their counterparts where LKB1 was reintroduced by retroviral transduction, H358 (wild-type LKB1) and HCC4017 (wild-type LKB1) cells (17). We found that PF-562,271 and 4 Gy of IR cause a comparable degree of upregulation of γ -H2AX. Furthermore, LKB1 did not influence the upregulation of γ -H2AX (Fig 3G and H; and Supplementary Fig. S3A–S3C). Taken together, our data support the conclusion that loss of FAK leads to a DNA damage response in mutant *KRAS* NSCLC cells.

FAK silencing or pharmacologic inhibition sensitizes oncogenic *KRAS* NSCLC cells to the effects of IRs

Our results suggest that FAK is required for promoting DNA damage repair in oncogenic *KRAS* NSCLC cells. Therefore, we tested whether pharmacologic inhibition of FAK sensitized NSCLC cells expressing oncogenic *KRAS* to the antiproliferative effects of IR. We performed clonogenic survival assays with H460, H358, H522, and H596 cells as representative examples of mutant and wild-type *KRAS* NSCLC cells, respectively, exposed to increasing doses of IR (1–6 Gy). We chose H358 cells for this experiment because they are LKB1 wild-type, while H460 and A549 are LKB1 mutant, to rule out the contribution of LKB1 loss to radiosensitization. We administered 1 μ mol/L PF-562,271, because we found that this concentration inhibits P-FAK in a comparable manner in the cells we used for this study (data not shown), 4 hours before exposure to IR. We limited the incubation time to 48 hours not to affect plating efficiency.

We found that pharmacologic inhibition of FAK with PF-562,271 to irradiated mutant *KRAS* H460 and H358 NSCLC cells resulted in profound changes in Dq as well as significant, but less dramatic, decreases in Do (Fig. 4A and B). Thus, we concluded that FAKi reduces inherent DNA repair capacity (Dq) as indicated by significant changes in the shoulder of the survival curves. In contrast, exposure of wild-type *KRAS* NSCLC cells (H522 or H596; Fig. 4C and D) to PF-562,271 had no significant effect on IR-induced lethality as noted by the survival curve and estimations of Dq and Do. Most importantly, the presence of FAKi increased dose enhancement ratios (DER) with values ranging between 2.1 and 1.9 at LD₅₀ levels, and between 1.7 and 1.4 at LD₂₀ levels, respectively (Supplementary Table S3).

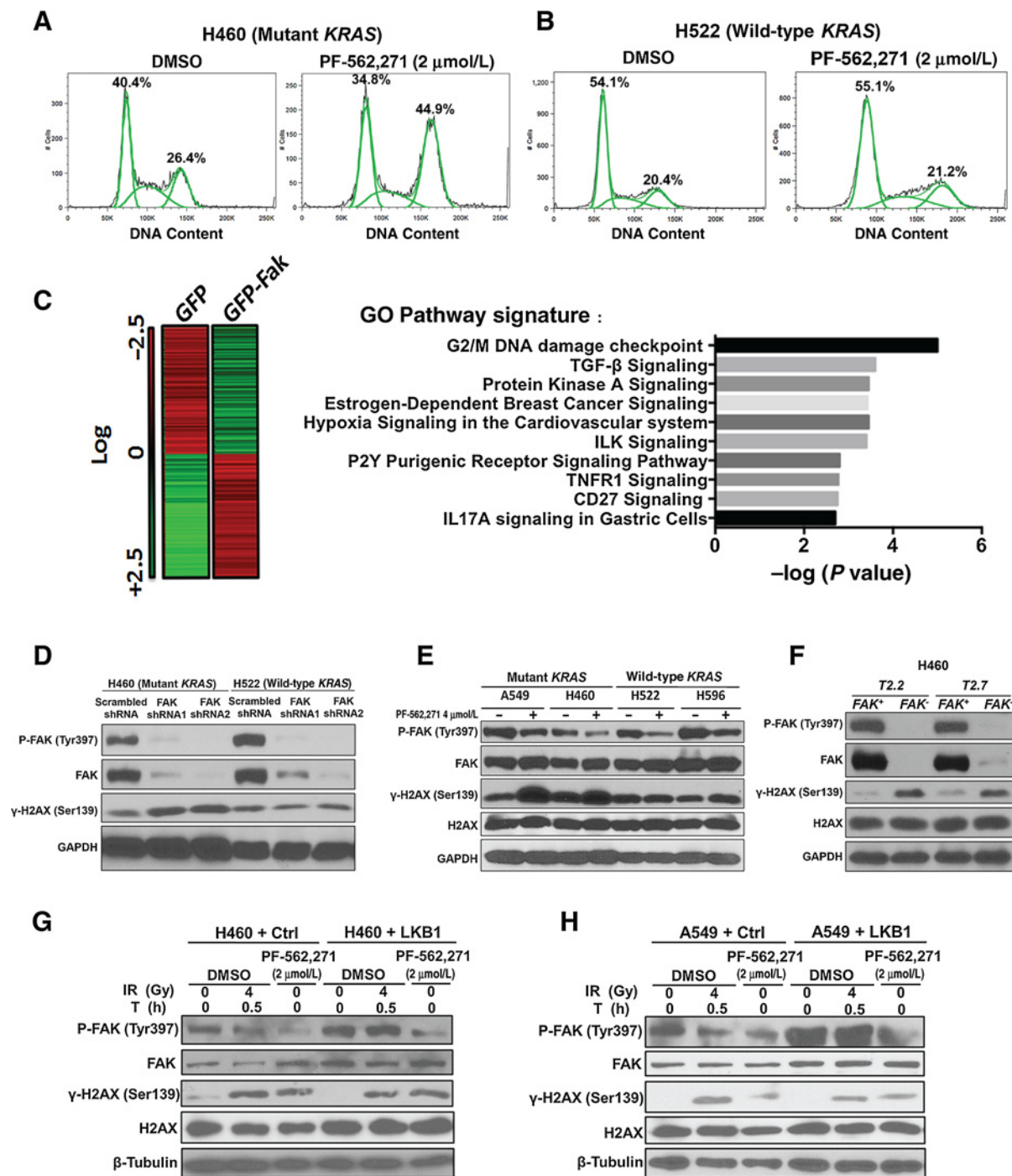
Notably, the effect of pharmacologic inhibition of FAK was comparable to the dual PI3K/mTOR inhibitor BEZ-235, which is a known radiosensitizing agent (Fig. 4A; refs. 20, 44).

To confirm these findings with an independent approach, we silenced FAK. Because prolonged silencing of FAK severely impairs clonogenic growth of mutant *KRAS* NSCLC cell lines, we used the GEPIR retrovirus to express FAK shRNA1 in a doxycycline-regulated manner, obtaining results equivalent to FAKi (Fig. 4E and F). We found that inducible silencing of FAK had effects similar to PF-562,271 treatment in both mutant and wild-type *KRAS* NSCLC cells.

These data indicate that silencing or pharmacologic inhibition of FAK inhibits the DNA repair and/or augment DNA damage created by IR.

Radiosensitization induced by FAK blockade or loss is accompanied by persistence of DNA damage foci

Our data suggest that inhibition of FAK facilitates the cytotoxic effects of IR by promoting increased or unresolved DNA damage. Thus, we examined whether PF-562,271 affects induction and repair of DNA breaks after IR exposure in H460 cells. We determined that resolution of γ -H2AX foci, a well-known marker of DNA double-strand break (DSB) damage and repair (when foci decrease), occurred rapidly after treatment with IR (2 Gy). In contrast, treatment with the FAK inhibitor PF-562,271 in combination with IR (2 Gy) led to a striking persistence of γ -H2AX foci at 24 hours after IR administration compared with exposure with IR alone. The effect of PF-562,271 in regard to inducing the persistence of γ -H2AX foci

**Figure 3.**

Suppression of FAK promotes a DNA damage response. **A** and **B**, Cell-cycle analysis of H460 and H522 cells treated as indicated. The percentage of cells in each phase of the cell cycle is indicated. Note a significant increase in the percentage of G₂ cells after treatment with FAKi. **C**, Heat maps of the genes enriched in a genome-wide expression profiling experiment, illustrating the changes in gene expression of HBEC cells expressing either pMXs-Puro-GFP or pMXs-Puro-GFP-Fak. Expression level shown is representative of \pm log (2.5) of each replicate ($n = 3$ samples/condition). Red signal denotes higher expression relative to the mean expression level within the group and blue signal denotes lower expression relative to the mean expression level within the group. The histogram shows Gene Ontology analysis of differentially expressed genes in enrichment analysis. **D**, WB analysis of H460 and H522 cells expressing the indicated shRNAs. Note upregulation of γ -H2AX in H460 cells with FAK knockdown. **E**, WB analysis of NSCLC cells treated with FAKi as indicated. Note upregulation of γ -H2AX in mutant, but not in wild-type, KRAS NSCLC cells treated with FAKi. **F**, WB analysis of H460 T2.2 FAK⁺ and FAK⁻ cells; note upregulation of γ -H2AX in FAK-null cells. **G** and **H**, WB analysis of H460 or A549 cells (both LKB1 mutant) stably transduced with either control or with LKB1 expressing retroviral vectors (H460 or A549 + control and H460 or A549 + LKB1, respectively). Cells were treated as indicated. Note that PF-562,271 cause upregulation of γ -H2AX to a degree comparable to IR in all cell lines.

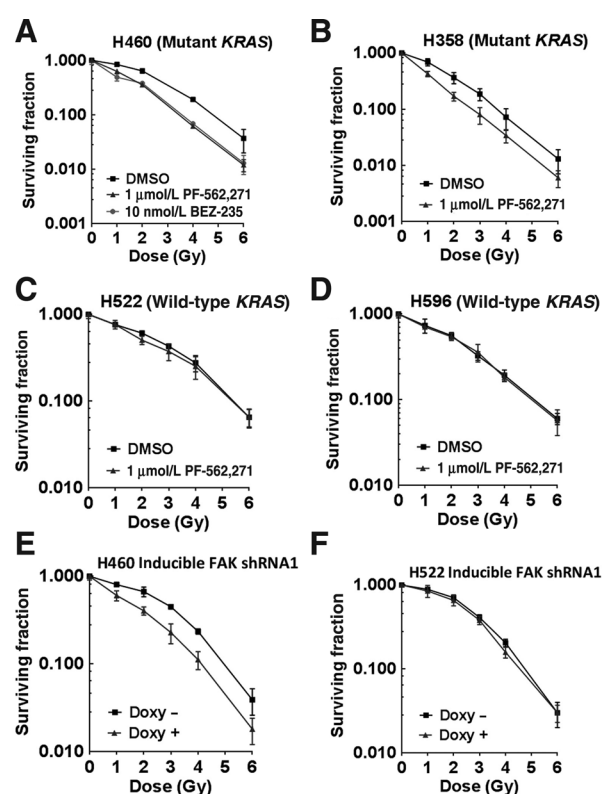


Figure 4.

FAK blockade sensitizes mutant *KRAS* NSCLC cells to the effects of IRs. **A–D**, Clonogenic survival assays of NSCLC cells treated as indicated. **E and F**, Clonogenic survival assays of H460 and H522 cells stably expressing a doxy-dependent FAK shRNA (shRNA1). Doxy turns on the shRNA. Doxy-treated cells are indicated. Colony number was calculated from three replicate plates of three independent experiments; bars, SD. Gy = Grey.

was comparable to the effects of the dual PI3K/mTOR inhibitor BEZ-235, a known radiosensitizer (Fig. 5A; refs. 20, 44). Inhibition of FAK in the absence of IR induces γ -H2AX foci slightly above the background level in this assay (Fig. 5A; Supplementary Fig. S4A). We obtained equivalent results in H358 and HCC4017 (LKB1 wild-type) cells (Supplementary Fig. S4B–S4E).

We also obtained equivalent results when we determined the induction and resolution of γ -H2AX and TP53BP1 foci, as readout of DNA damage, in CRISPR/CAS9 H460 T2.2 FAK⁺ and H460 T2.2 FAK[−] cells (Fig. 5B; Supplementary Fig. S5A).

Next, we confirmed by flow cytometry that inhibition of FAK with PF-562,271 or in FAK null T2.2 H460 cells affects the percentage of γ -H2AX-positive cells after exposure to IR at every time point tested (Supplementary Fig. S5B and S5C). We found that mutant *KRAS* H460 cells treated with FAKi display a significant increase of the percentage of cells in the G₂ phase of the cell cycle, which was further increased 4 and 24 hours after combination treatment with FAKi and IR as compared to H460 cells treated with IR only (Fig. 5C).

Western blot analysis confirmed that PF-562,271 increases γ -H2AX and in combination of IR leads to further upregulation and persistence of γ -H2AX in H460 cells (Fig. 5D). Furthermore, we found that IR activates P-FAK, which persists for at least 8 hours

after IR (Fig. 5D). In addition, cell fractionation experiments revealed that a portion of FAK resides in the cell nucleus where DNA repair takes place (Supplementary Fig. S5D). FAK-null H460 cells not only display an increased basal level of γ -H2AX and its further upregulation after IR treatment, as compared to wild-type FAK H460 cells, but also persistent activation of CHK2 after exposure to IR (Fig. 5E). We detected similar findings with respect to γ -H2AX in H460 cells that underwent FAK inhibition or CRISPR/CAS9-mediated ablation, with only minor differences in the kinetics of resolution of γ -H2AX activation at later time points (Fig. 5D and E; and Supplementary Figs. S4A, S5A–C, and S5E). In this regard, there were small incongruences between the intensities of the signals obtained by IF, WB, and flow cytometry, which may be due to the fact that WB detects total cellular γ -H2AX while IF detects γ -H2AX accumulated in foci, which are also more readily detected by flow cytometry. Because γ -H2AX is a marker of DNA damage when it is accumulated in nuclear foci, we conclude that the presence of nuclear foci is representative of ongoing DNA damage.

Taken together, these data suggest that inhibition/suppression of FAK results in persistent DNA damage in NSCLC cells because of inhibition of DNA repair or augmentation of damage by cell-cycle checkpoint abrogation, which occur without affecting the activation and recruitment to sites of DNA damage of the DNA damage-sensing machinery. These observations also suggest that IR therapy could be exploited to sensitize cancer cells to therapy with FAK inhibitors.

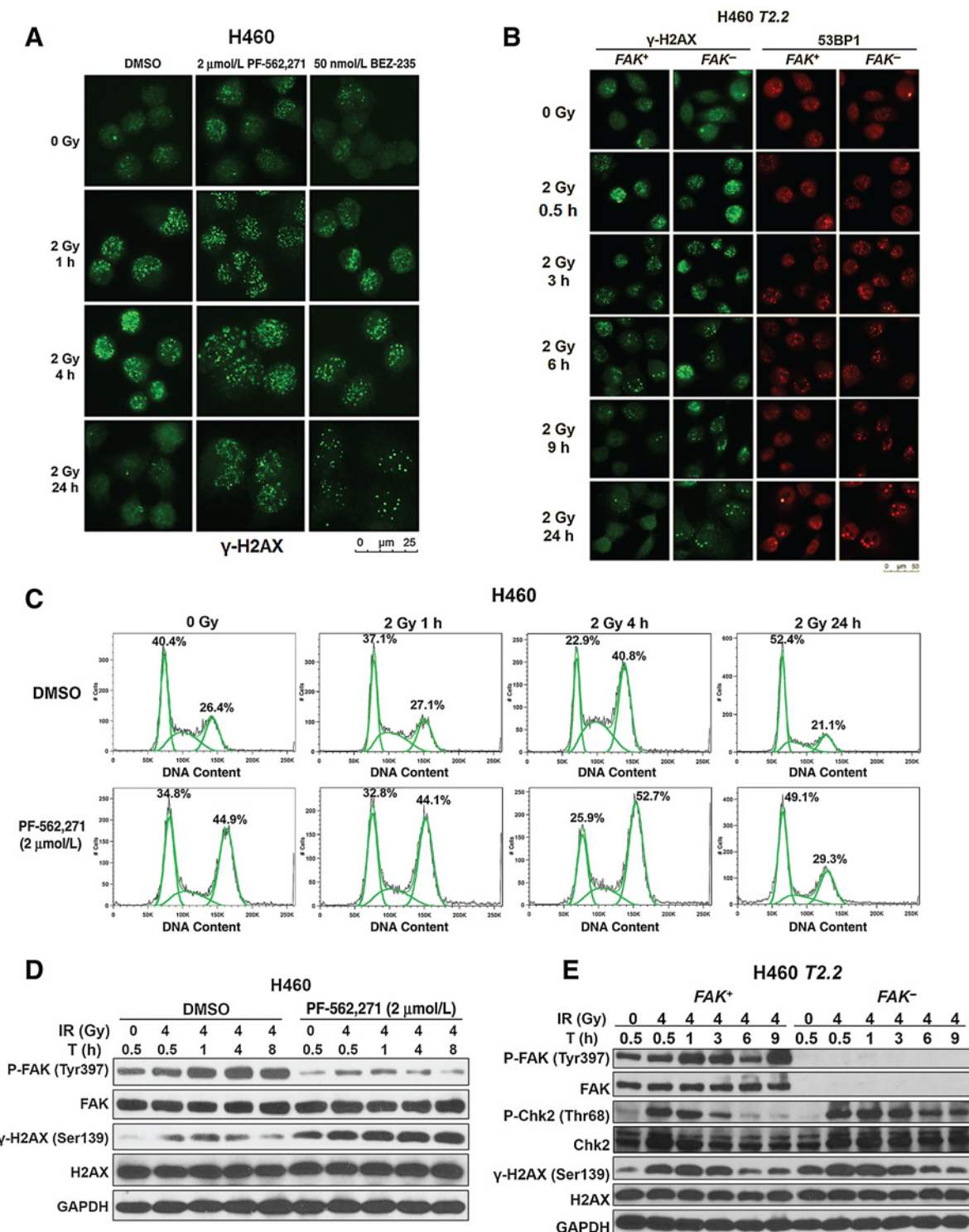
Combination of FAK inhibition and radiotherapy is an effective antitumor strategy *in vivo*

We tested the antitumor effects of FAK inactivation mediated by CRISPR/CAS9 editing in combination with IR in H460 NSCLC xenografts, a well-established model of aggressive NSCLC and a representative example of the cells we used in tissue culture experiments.

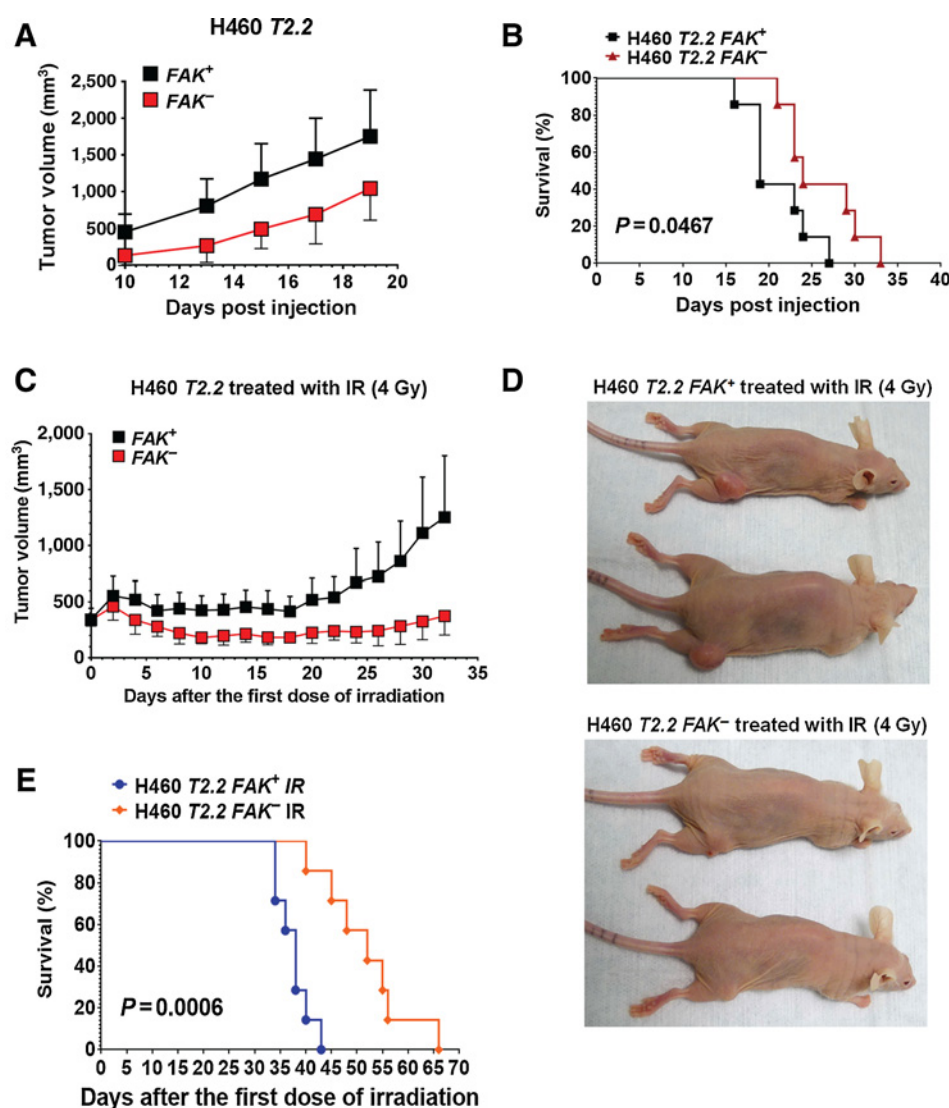
We generated four cohorts of seven athymic nude mice bearing xenografts of H460 T2.2 FAK⁺ and H460 T2.2 FAK[−] cells of 300-mm³ average size. Mice were either mock treated or treated with five 4-Gy fractions every other day for 10 days. We delivered local irradiation to the flank or thigh of lead-shielded mice using a fractionated dose to limit overall tissue toxicity and mimic the administration modality used in the clinic (45). Notably, this xenograft volume and IR dose is comparable with previous studies involving H460 xenografts (20, 46).

As expected, FAK ablation impaired xenograft growth compared to the parental H460 cells (Fig. 6A). The median survivals of mock-treated mice carrying H460 T2.2 FAK⁺ and FAK[−] cells were 19 days and 24 days, respectively ($P = 0.0467$; Fig. 6B). IR treatment of H460 T2.2 FAK[−] cells resulted in a greater than 75% reduction in xenograft volume as compared with H460 T2.2 FAK⁺ cells 30 days after the first dose of IR ($P < 0.001$; Fig. 6C and D). All but one of the irradiated mice carrying xenografts of H460 T2.2 FAK[−] cells were alive 43 days after the initiation of IR treatment; in contrast, every mouse carrying xenografts of H460 T2.2 FAK⁺ cells was sacrificed between days 34 and 43 after radiation due to excessive tumor burden (Fig. 6E).

H460 T2.2 FAK[−] cells resumed their growth about 40 days after the first dose of radiation was administered. Xenograft growth eventually reached the maximum size allowed in these experiments, and mice had to be sacrificed. By IF analysis of

**Figure 5.**

Radiosensitization induced by FAK blockade is accompanied by persistence of DNA damage foci. **A**, Detection by immunofluorescence of γ -H2AX foci in H460 cells treated with DMSO, PF-562,271, or BEZ-235 followed by 2 Gy of IR. Foci were detected at the indicated time points. Note striking increase in the number of foci 24 hours after treatment with PF-562,271 and BEZ-235 (a known radiosensitizing drug) in combination with IR. Bar, 25 μ m. **B**, Detection by immunofluorescence of γ -H2AX foci in T2.2 FAK⁺ and FAK⁻ H460 cells after treatment with 2 Gy of IR. Foci were detected at the indicated time points. Note striking increase in the number of foci at 9 and 24 hours after IR. Bar, 25 μ m. **C**, Cell cycle analysis of H460 cells treated as indicated. The percentage of cells in each phase of the cell cycle is indicated. Note a significant increase in the percentage of G₂ cells after combination treatment with FAKi and IR. **D**, WB of H460 cells treated with PF-562,271 as indicated. **E**, WB of T2.2 FAK⁺ and FAK⁻ H460 cells after IR.

**Figure 6.**

Loss of FAK is radiosensitizing in a xenograft tumor model. **A**, Xenograft growth of H460 T2.2 FAK⁺ and FAK⁻ cells in nude mice treated as indicated. The graph shows xenograft volumes. Points represent the mean of tumor volume (mm³) at each time point. **B**, Kaplan-Meier curve of xenografts of H460 T2.2 FAK⁺ and FAK⁻ cells. $P = 0.0467$. **C**, Xenograft growth of H460 T2.2 FAK⁺ and FAK⁻ cells in nude mice treated with IR as indicated. **D**, Tumor burden in representative mice carrying xenografts of the indicated genotype 20 days after initiation of IR treatment. **E**, Kaplan-Meier curve of xenografts of H460 T2.2 FAK⁺ and FAK⁻ cells treated with IR as indicated. $P = 0.0006$. Number of mice = 7/group; mice were sacrificed when tumor volume reached 2,000 mm³; bars, SE; P value is indicated.

frozen sections we determined that T2.2 FAK⁻ cells remained negative for FAK at the experimental endpoint (Supplementary Fig. S5F). This finding suggests that FAK-independent mechanisms may mediate resistance to FAK inhibition. It is possible that other tyrosine kinases or other prosurvival networks may mediate resistance to FAK inhibition. IR treatment was well tolerated in xenograft bearing nude mice and we did not observe any drop in body weight or other signs of toxicity in both groups (data not shown).

These results indicate that the ablation of FAK leads to significant radiosensitizing effects, which in turn led to significant antitumor effects *in vivo*.

Discussion

NSCLC remains a significant clinical challenge due to the fact that few medical treatments are effective in this disease. It is well known that NSCLC displays either primary or acquired resistance to chemotherapy and targeted therapy. The limitations of current therapies are evident in mutant KRAS

NSCLC. For instance, direct inhibitors of oncogenic KRAS lack the specificity needed for their deployment *in vivo* (47–49). Furthermore, inhibition of the canonical KRAS signaling pathways MEK1/2, PI3K, and mTORC1/2 have not shown benefits in lung cancer patients that justify their FDA approval for clinical use (4). As a consequence, there has been an intense interest in the identification of novel therapeutic targets for mutant KRAS NSCLC.

The data presented in this article lead to several important conclusions: not only is FAK a targetable vulnerability of mutant KRAS lung cancer, but its inhibition also leads to significant radiosensitizing effects that can be exploited in a combination therapy regimen. This finding is of significance because of the availability of several FAKi in clinical development.

We previously reported a mutant KRAS–RHOA–FAK signaling axis in NSCLC and that both RHOA and FAK are requirements of high-grade NSCLC (8). Our analysis in a large panel of lung cancer cells confirms and extends our prior findings obtained with RNAi in a smaller sample set of NSCLC cells and in a genetically engineered mouse model of lung cancer

(8). Our new set of data is of significance because we used specific FAKi PF-562,271 (the parental compound of Vs6063, also known as defactinib) and VS-4718. Both defactinib and VS-4718 are being evaluated in clinical trials in lung cancer and mesothelioma. Furthermore, we used shRNAs and gene editing, which are complementary and nonoverlapping methods to genetically inactivate *FAK*. Thus, we reason that by using these complementary approaches we adequately addressed the concern that the interpretation of RNAi experiments may be hampered by off-target effects (50). Notably, the results we obtained are internally consistent, indicating that the deleterious effects on cell proliferation, clonogenic capacity, and delayed xenograft growth *in vivo* are caused by *FAK* inhibition.

Even though we tested NSCLC cells carrying oncogenotypes other than mutant *KRAS*, we did not find antiproliferative responses consistently in wild-type *KRAS* NSCLC cells. Thus, we conclude that the dependency on *FAK* is a specific feature of mutant *KRAS* NSCLC cells. Accordingly, mutant *KRAS* status could be used as a biomarker for the enrolment of patients in clinical trials using *FAK* inhibitors.

Our data indicate that *FAK* is implicated in the response to DNA damage in mutant *KRAS* NSCLC cells. Pharmacologic inhibition of *FAK* protein, *FAK* gene silencing, or ablation results in activation and maintenance of a DNA damage response as demonstrated by the presence of γ -H2AX in Western blots and the recruitment of γ -H2AX and TP53BP1 to DNA damage foci. In this regard, it is also noteworthy that it was reported that *FAK* loss in murine breast cancer cells and primary fibroblast is associated with induction of replicative senescence, even though the mechanism underlying this outcome was not defined (25). In addition, *FAK* inhibition was implicated in mediating radiosensitization in several other settings, for instance, melanoma and head and neck cancers (51, 52).

In xenograft experiments with *FAK*-null lung cancer cells, we noted that the impairment of the growth of *FAK*-null xenografts declined over time. The observation that *FAK*-null xenografts stain negative for *FAK* at the experimental endpoint suggests that *FAK*-independent mechanisms mediate resistance to *FAK* inhibition. It is possible that other tyrosine kinases or other prosurvival networks may mediate this phenomenon. The identification of mechanisms that mediate resistance to *FAK* inhibition is the subject of ongoing investigations in our lab.

Our report also provides the first demonstration that the *FAK* blockade is an effective strategy to sensitize mutant *KRAS* NSCLC cancer cells to the deleterious effects of IR both in clonogenic assays and *in vivo*. In this regard, it is also possible that IR, by potentiating the therapeutic effects of FAKi, may delay or prevent the emergence of drug resistance.

These findings suggest that *FAK* blockade/ablation in combination with IR both inhibits inherent DNA repair [i.e., decreased shoulder (Dq) values] in oncogenic *KRAS*-driven NSCLC, as well as augments DSBs (noted by decreased Do values, representing the number of hits needed to cause death, high-dose enhancement ratios), consistent with enhanced formation of foci due to DNA DSBs. These interpretations are consistent with the observation that the DNA damage foci induced by IR persist in *FAK*-deficient cells. In this setting, decreased clonogenic capacity may be caused by growth arrest or impaired cell proliferation, inhibition of prosurvival stress responses or mitotic catastrophe. Indeed, we found that inhibition or loss of *FAK* leads to a G₂ cell-cycle arrest, which is

further increased by IR treatment. Thus, it is likely that the effects of *FAK* inhibition/suppression are mediated by loss of G₂ checkpoint control that, in turn, leads to an inherent loss of DNA repair capacity. Alternatively, these effects could be caused by inhibition of a DSB repair protein (such as ATM, ATR, or DNA-PKcs) that affects DSB repair and cell-cycle checkpoint control. In this respect, it is noteworthy that PF-562,271 exposure phenocopies the effects of the dual PI3K/mTOR inhibitor BEZ-235, which was reported to be an inhibitor of ATM and DNA-PK (20, 44). In the future, it will be of interest to differentiate between these two possibilities.

We noted for the first time that IR activates *FAK* and that *FAK* depletion promotes the hyperactivation of checkpoint kinase CHK2, a known downstream target of ATM/ATR and G₂-M effectors of cell-cycle arrest or apoptosis in response to DNA damage.

In this respect, it is noteworthy that *FAK* was reported to interact in the nucleus with PIAS1, a SUMO E3 ligase that interacts with BRCA1 in the DNA damage response (53–55). Thus, it is tempting to speculate that the requirement for *FAK* in mutant *KRAS* NSCLC cells is due to the fact that in basal growth conditions *FAK* promotes the repair of the DNA damage caused by oncogenic *KRAS* activation and that the requirement for *FAK* is further magnified in cells exposed to IR. In addition, p53, CDKN2A, and ATM tumor suppressors, which are well-known players in the DNA damage response and are also frequently comutated with mutant *KRAS*, may collaborate with *FAK* either directly or indirectly in mediating DNA damage repair (2, 3, 56, 57). The results of the experiments with H460 and A549 cells with restored LKB1 lead to the conclusion that loss of LKB1, another tumor suppressor implicated in DNA damage response, does not determine the dependency on *FAK*, at least in this context (43).

The FAKi defactinib and VS-4718 are being tested in phase I/II clinical trials, including lung cancer (58). Therefore, our work provides preclinical data useful for the design of therapeutic protocols using this novel class of drugs. We propose that mutant *KRAS* should represent a biomarker for the enrollment of patients in clinical trials using FAKi in NSCLC. Furthermore, we suggest that the clinical testing of combined therapy using FAKi and IR should be prioritized. In the future, it will be of interest to determine the mechanistic underpinning of the role of *FAK* in DNA damage repair to optimize the use of FAKi in cancer patients, identify biomarkers that predict clinical response and test FAKi in other tumor types that harbor mutant *KRAS*.

Disclosure of Potential Conflict of Interest

J.V. Heymach reports receiving other commercial research support from AstraZeneca, Bayer, and GlaxoSmithKline; and is a consultant/advisory board member for Ariad, AstraZeneca, Boehringer Ingelheim, Exelixis, Genentech, GlaxoSmithKline, Lilly, Novartis, and Synta. No potential conflicts of interest were disclosed by the other authors.

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PIAS1-FAK Interaction Promotes the Survival and Progression of Non-Small Cell Lung Cancer



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Abstract

The sequence of genomic alterations acquired by cancer cells during tumor progression and metastasis is poorly understood. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that integrates cytoskeleton remodeling, mitogenic signaling and cell survival. FAK has previously been reported to undergo nuclear localization during cell migration, cell differentiation and apoptosis. However, the mechanism behind FAK nuclear accumulation and its contribution to tumor progression has remained elusive. We report that amplification of *FAK* and the SUMO E3 ligase *PIAS1* gene loci frequently co-occur in non-small cell lung cancer (NSCLC) cells, and that both gene products are enriched in a subset of primary NSCLCs. We demonstrate that endogenous FAK and PIAS1 proteins interact in the cytoplasm and the cell nucleus of NSCLC cells. Ectopic expression of *PIAS1* promotes proteolytic cleavage of the FAK C-terminus, focal adhesion maturation and FAK nuclear localization. Silencing of *PIAS1* deregulates focal adhesion turnover, increases susceptibility to apoptosis *in vitro* and impairs tumor xenograft formation *in vivo*. Nuclear FAK in turn stimulates gene transcription favoring DNA repair, cell metabolism and cytoskeleton regulation. Consistently, ablation of *FAK* by CRISPR/Cas9 editing, results in basal DNA damage, susceptibility to ionizing radiation and impaired oxidative phosphorylation. Our findings provide insight into a mechanism regulating FAK cytoplasm-nuclear distribution and demonstrate that FAK activity in the nucleus promotes NSCLC survival and progression by increasing cell-ECM interaction and DNA repair regulation.

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Introduction

Protein inhibitor of activated STAT1 (*PIAS1*) is a SUMO E3 ligase implicated in the regulation of several oncogenes and tumor suppressors such as AKT, BRCA1, BRCA2, PML and PML-RARA [1–4]. In addition, *PIAS1* is over-expressed in prostate and lung cancers [4,5]. Moreover, increase in PIAS1 protein levels has recently been linked to breast cancer tumorigenesis, albeit reports disagree as to the relevance of

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PIAS1 to tumorigenesis and metastasis [6,7]. Thus, we decided to characterize the relevance of PIAS1 in non-small cell lung cancer (NSCLC) progression and metastasis. We also investigated PIAS1 downstream targets that could account for the phenotype observed and potentially serve as a therapeutic target in NSCLC.

Lung cancer metastasis is an indicator of poor prognosis and a main determinant of cancer-related mortality. Consequently, targeting and prevention of cancer cell metastasis is among the biggest hurdles in clinical oncology [8]. During metastasis, cancer cells rely heavily on cell-extracellular matrix (ECM) interactions, cytoskeleton remodeling and gene transcription. An important player in these processes is focal adhesion kinase (FAK). FAK is a non-receptor tyrosine kinase that contributes to almost every aspect of metastasis; from ECM sensing, cytoskeleton remodeling to gene transcription [9–12]. The *FAK* gene is rarely mutated in human lung cancers, but the *FAK* locus (chromosome 8q) is frequently amplified in lung, colon, breast and gastric tumors [13–16].

FAK controls cytoskeleton remodeling by transducing signals from integrin receptors to ERK/MAPK, PI3K, RAC1 and RHOA [10,17–19]. Importantly, FAK promotes integrin $\beta 1$ (ITG $\beta 1$) gene expression, which in turn, increases the survival of cancer cells [20]. FAK has also been linked to transcriptional activation of *SNAIL*, *TWIST*, *ZEB1*, and *ZEB2* genes, which are essential for epithelial to mesenchymal (EMT) reprogramming in epithelial cells [21–23]. However, whether FAK is involved in transcriptional regulation is still a matter of debate because FAK resides mainly in the cytoplasm where it is associated with the plasma membrane. However, FAK protein can relocate to the cell nucleus during cell differentiation or cancer progression [24,25]. Despite several studies reporting FAK protein nuclear localization and involvement in gene transcription, no unifying mechanism exists to explain the nuclear accumulation of FAK and the potential implications of nuclear FAK for tumorigenesis and metastasis.

Small ubiquitin-like modifiers (SUMO) have recently gained attention because of their participation in the covalent modification of target protein substrates, a process referred to as SUMOylation. This process consists of an enzymatic cascade whereby SUMO proteins are added onto target substrates with the involvement of E1, E2 and a limited number of SUMO E3 ligases. Typically, only a small fraction of a given protein is SUMOylated [26]. SUMOylation has been implicated in several cellular processes that include the regulation of nuclear import, DNA damage repair and signal transduction, however its role in tumorigenesis is still incompletely understood [27].

Using single nucleotide polymorphism (SNPs) data, we discovered that *PIAS1* and *FAK* are frequently co-amplified in lung cancer specimens. We found a positive correlation between increased gene copy number and FAK and PIAS1 protein levels in a subset of NSCLC cell lines *in vitro*, human lung tumor samples *in vivo* and in a mouse model of tumor metastasis. Herein, we report an interaction between FAK-PIAS1 leads to FAK nuclear relocation, which is crucial for the regulation of the turnover of focal adhesions, and cell survival during oncogenic stress.

Materials and Methods

Gene Copy Number Analysis

SNP was performed as previously described [4]. Briefly, SNP profile was obtained using Illumina DNA analysis Bead Chip (Illumina, Inc.). *PIAS1* and *FAK* gene copy number was extrapolated from their relative probe intensity compared with diploid controls.

Histochemistry and Immunofluorescence

Formalin-fixed, paraffin-embedded NSCLC specimens were obtained from the human tumor tissue bank at UT Southwestern Medical Center. Tissues and cells were processed for immunohistochemistry (IHC) or immunofluorescence (IF) using standard protocols [19]. See also supplementary methods.

Cell Lines and Tissue Culture

Human NSCLC cells and human bronchoalveolar epithelial cells (HBECs) were from the Hamon Center Cell Line Repository and were a gift of Dr. John Minna (UT Southwestern Medical Center) [28,29]. All cell lines were DNA-fingerprinted for provenance (PowerPlex 1.2 Kit; Promega) and Mycoplasma-free (e-Myco Kit; Boca Scientific). NSCLC cells were cultured in RPMI supplemented with 5% serum and antibiotics. HBEC cells were grown in keratinocyte growth media with supplements (Invitrogen, No.: 17005-042). NIH 3T3 were grown in 5% DMEM. All cells were maintained in humidified incubator with 5% CO₂ at 37°C. See also supplementary methods.

Western Blotting and Immunoprecipitation

We followed standard protocols [19].

Soft Agar Colony Formation, Transwell Migration and Scratch Assays

We performed these assays following established procedures [11,30].

Subcellular Fractionation

Cellular fractionation was carried out as previously described [31]. Cells were scraped with ice-cold PBS, pelleted by centrifugation at 600 g for 5 minutes, suspended in hypotonic lysis buffer (10 mM Tris-base pH 7.5, 10 mM KCl, 1.5 mM MgCl₂) with protease/phosphatase inhibitors for 10 minutes. Cells were then lysed by Dounce homogenization and centrifuged at 3000×g to separate cytoplasmic (supernatant) from nuclear (pellet) fractions. To extract nuclear proteins, the nuclear pellet was resuspended in high salt buffer (50 mM Tris-base, pH 7.9, 0.5 M NaCl) 2 mM EDTA, 10% sucrose, 10% glycerol) with protease and phosphatase inhibitors.

RNAi Interference

Stable lentiviral transduction was performed with pGIPZ Lentiviral vectors containing shRNAs targeting *PIAS1* and non-targeting scrambled shRNA controls (Dharmacon). siRNA (siGenome) targeting *PIAS1*, or non-targeting siRNA controls were purchased from Dharmacon. See also supplementary methods.

Oxygen Consumption Rate Measurement

This assay was performed with a Seahorse XF analyzer following the manufacturer's protocols (Seahorse Bioscience).

Mouse Xenografts

Xenografts were performed as described previously [32] and were approved by the institutional IACUC guidelines.

Results

FAK and PIAS1 Genes are Frequently Co-Amplified in a Subset of NSCLCs

Using SNPs gene copy number analysis we looked for novel genomic cooperating alterations in a panel of 108 non-small cell lung cancer (NSCLC) cells. This analysis revealed that approximately 8%

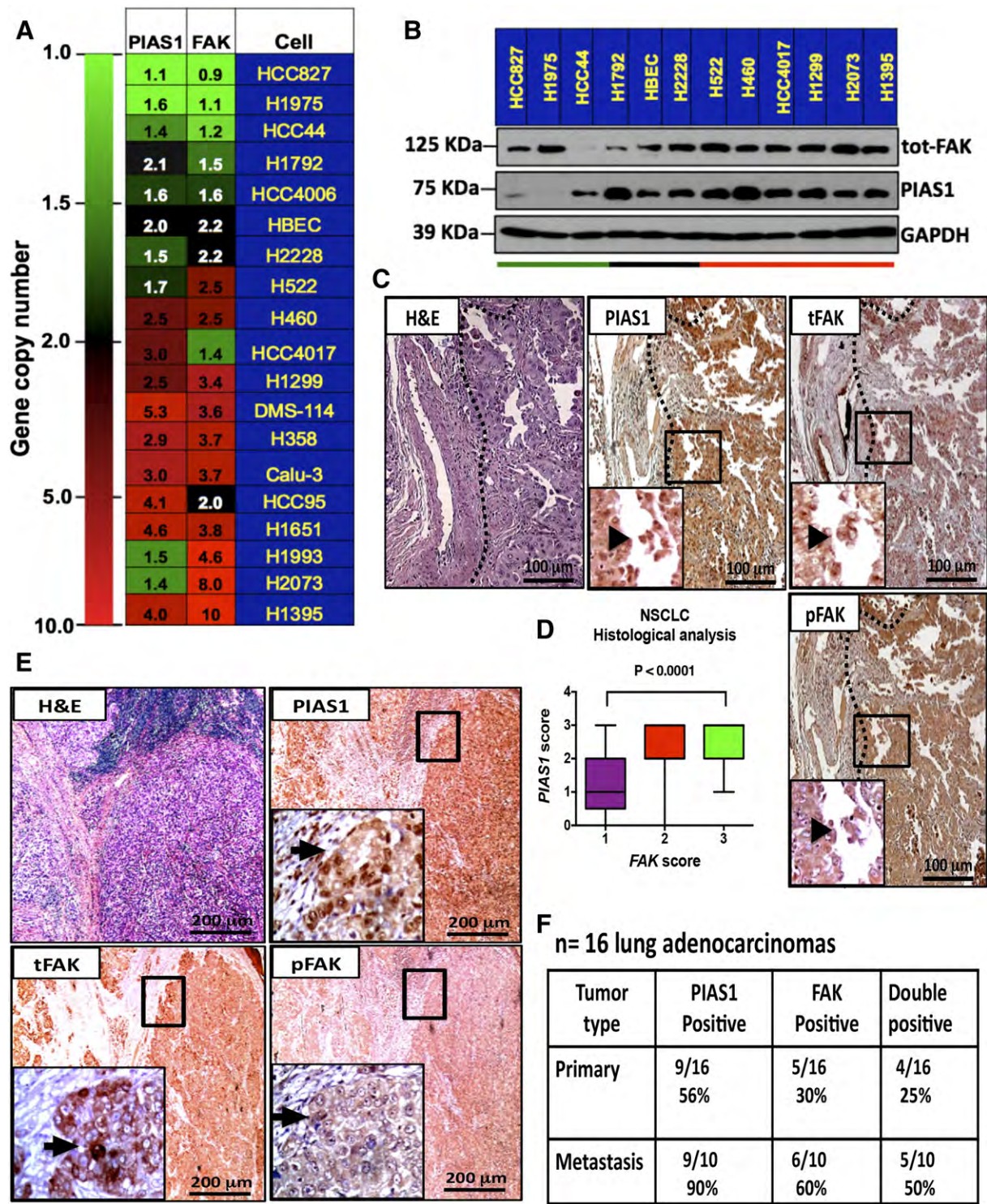


Figure 1. FAK and PIAS1 protein levels are correlated in NSCLC. (A) Heatmap shows single nucleotide polymorphism (SNPs) gene copy number analysis for *FAK* and *PIAS1* genes in the indicated lung cancer cell lines. Green, black and red indicate copy number loss, no change or gene copy number gain, respectively. (B) Immunoblot confirms increment in FAK and PIAS1 at the protein level in a representative panel of cells with gene amplification. Green, black and red horizontal lines indicate NSCLC cells with loss, no change or gene copy number gain of *PIAS1* and *FAK*. (C) Hematoxylin and eosin (H&E) and IHC stain of PIAS1, phospho-FAK (pFAK) and total FAK in primary NSCLC samples. Insert shows cytoplasmic and nuclear positivity for both proteins (black arrowheads). Scale bar: 100 μ m. (D) IHC positivity score analysis of FAK and PIAS1 in human NSCLC tumor tissue microarrays samples (n = 330). Graph shows positive correlation between increase positivity in FAK stain and PIAS1. Score = 1 low intensity; 2 = moderate intensity; 3 = high intensity. Statistical analysis was done using a Mann-Whitney U test. (E) H&E and IHC stain of PIAS1, phospho-FAK (pFAK) and total FAK in metastatic NSCLC. Insert shows positivity for cytoplasmic and nuclear stain for both proteins. Scale bar: 200 μ m. (F) Contingency table shows positive correlation between FAK and PIAS1 IHC stain positivity in primary vs metastatic samples. The number of samples is indicated.

(ie, 12 cell lines) of NSCLC cells had amplification of the *FAK* and/or *PIAS1* genes (Figure 1A).

To investigate the relationship between gene copy number gain and gene expression we detected FAK and PIAS1 by immunoblot in cell lines representative of our larger panel of NSCLC cells. We found that cell lines with increased PIAS1 gene copy number display concordant increase in PIAS1 protein (Figure 1B). Since gene amplification/deletions may result from adaptive responses to selective pressures of tissue culture, we tested primary NSCLC specimens for FAK and PIAS1 proteins using IHC. We found that primary NSCLC samples exhibited elevated total FAK (tot-FAK), phospho-FAK (p-FAK; Y397) and PIAS1 positivity compared to normal surrounding tissue (Figure 1C, arrowhead). Using lung tumor tissue microarrays we further confirmed that samples with high tot-FAK positivity score had a concomitant increase in PIAS1 positivity, but not p-FAK positivity (Figure 1D). Because FAK is associated with cell migration [9,33], we tested paired primary and metastatic NSCLC samples for FAK and PIAS1 protein levels. As observed in primary lung tumor samples, we found a positive correlation with FAK and PIAS1 protein levels in lymph node metastasis, but not with p-FAK protein levels (Figure 1, E and F). These observations suggest that PIAS1 and total-FAK protein levels correlate with tumor progression, independent of FAK phosphorylation.

The *Kras*^{G12D}/*p53*^{R172H} mouse model (KP hereafter) faithfully recapitulates aggressive human lung adenocarcinoma, developing advanced lung tumors that are highly metastatic [34,35]. Metastatic lung tumors in this mouse model have been fully characterized and show elevated expression of the *ZEB1* transcription factor and activation of Notch1 and Jagged2 signaling pathways, recapitulating the EMT profiles associated with human metastatic NSCLC [36,37]. As in human NSCLC, we found that KP lung tumors are positive for total-FAK and PIAS1, but not for p-FAK (Supplementary Fig. 1A). Next we took advantage of 393P and 344SQ lung cancer lines, which are a pair of lung cancer cell lines derived from a KP lung specimen. 393P cells, were derived from a primary lung tumor, are epithelial and have low metastatic potential; while 344SQ cells were derived from a metastatic KP lung tumor, are mesenchymal and are highly metastatic [34]. In this experimental system we found that PIAS1 is significantly up-regulated *in vivo* and metastatic cell line 344SQ (Supplementary Fig. 1B).

In view of these findings, we concluded that co-amplification of *FAK* and *PIAS1* genes occurs in a subset of primary human NSCLC samples. Moreover, subsets of primary and metastatic NSCLC samples have elevated PIAS1 and tot-FAK protein levels.

PIAS1 and FAK Proteins Physically Interact in NSCLC

We tested whether FAK and PIAS1 physically interact with co-IP assays using NIH-3T3 cells transiently transfected with *FAK* or *PIAS1* cDNAs. We successfully co-immunoprecipitated FAK with PIAS1 from transfected NIH-3T3 cells (Figure 2A). Furthermore, we co-immunoprecipitated endogenous FAK and PIAS1 proteins in several NSCLC cells, which co-amplify *FAK* and *PIAS1* genes (Figure 2B).

To gain insights into the location and stimulus required for FAK and PIAS1 interaction, we performed cellular co-fractionation of endogenous proteins during serum starvation, apoptosis or mitogenic stimulation. To our surprise, we discovered that FAK and PIAS1 mostly co-purified in the cytosolic fractions, when cells were treated with protein kinase C inhibitor (PKCi: Calphostin-C, a proapoptotic agent), or serum for 2–4 hours post starvation, but not after stimulation with epithelial growth factor (EGF) (Figure 2C lanes 5, 7 and 10). Using IF and confocal microscopy we found significant co-localization of

endogenous FAK and PIAS1 at the nuclear periphery of serum treated cells (Figure 2D). We subsequently performed immunoblot and confocal co-localization studies with well-characterized organelle markers following serum stimulation. Although partial localization of PIAS1 and FAK can be seen with the endoplasmic reticulum protein Calnexin, the dominant sites of interaction contain the RAB11 and LAMP1, endosome associated proteins. These findings suggest that internalization of growth factor receptors complexes promote the association of FAK and PIAS1 proteins in endosomes localized in the cytoplasm and nuclear periphery (Supplementary Fig. 2A–C).

To begin testing the biological significance of the interaction between FAK and PIAS1, we determined the effects of *Flag-Pias1* and or SUMO overexpression on FAK proteins. We found that overexpression of *Flag-Pias1* or SUMO-1 did not promote a significant change in tot-FAK or p-FAK (Figure 2E). However, expression of *Flag-Pias1* or the combination of *Flag-Pias1* and SUMO1 resulted in the appearance of a lower molecular weight species of FAK (Figure 2E arrowheads).

FAK was previously reported to undergo proteolytic cleavage by Calpain proteases on its C-terminus to allow disengagement from focal adhesions [38]. To test whether PIAS1 contributes to FAK C-terminal cleavage we used a mutant (*Fak*^{V744G}) that abolishes FAK protein C-terminal cleavage. Indeed, we found that the *Fak*^{V744G} is resistant to PIAS1-induced FAK C-terminal proteolysis (Figure 2F).

Thus, we concluded that mitogenic signaling promotes FAK and PIAS1 physical interaction, which correlates with PIAS1-induced FAK C-terminal cleavage. Importantly, this phenotype is rescued by using a *FAK* mutant resistant to proteolytic cleavage.

PIAS1-FAK Interaction Regulates Focal Adhesion Dynamics

Endogenous PIAS1 is mostly nuclear, but can also be found scattered in the cytoplasm of lung cancer cells *in vivo* and *in vitro* (Figures 1C and 2D). We hypothesized that PIAS1-FAK interaction may affect focal adhesion dynamics and how cells integrate extracellular signaling. To test this hypothesis, we used RNAi to determine how *PIAS1* affects F-Actin stress fiber formation. We found that *PIAS1* silencing in lung cancer cells destabilizes F-Actin fibers and reduces Vinculin (VCL) localization to focal adhesions (Figure 3A). Then we tested if the opposite was true using HBECs, which are immortalized, but not transformed, by transduction of CDK4 and human Telomerase Reverse Transcriptase (hTERT) [29]. Indeed, we found that ectopic expression of *PIAS1* (*Flag-Pias1*) in HBECs increases VCL puncta at focal adhesions, while also promoting the nuclear localization of GFP-FAK (*GFP-Fak*) (Figure 3B). We further tested for changes in VCL puncta formation in live cells using mCherry labeled VCL (*mCherry-VCL*). We measured the formation, duration and turnover of mCherry-VCL focal adhesion, as inverse correlation to FAK-dependent turnover of focal adhesions [11,38]. Compared to control shRNA, *PIAS1* silencing increased the turnover of mCherry-VCL puncta; along the leading and lagging edge of cells (Figure 3, C–F). Furthermore, we found that *Flag-Pias1* gene expression induces membrane ruffle formation in fibroblasts and rescues endogenous VCL puncta loss in *GFP-Fak* overexpressing cells (Supplementary Fig. 3A, B). Finally, induction of membrane ruffles by PIAS1 correlates with increases in Rac1-GTPase and ROCK-1 protein levels without an increase in pFAK levels (Supplementary Fig. 3C).

In view of these findings we concluded that PIAS1 regulates focal adhesion dynamics by promoting FAK nuclear localization and directional cell motility.

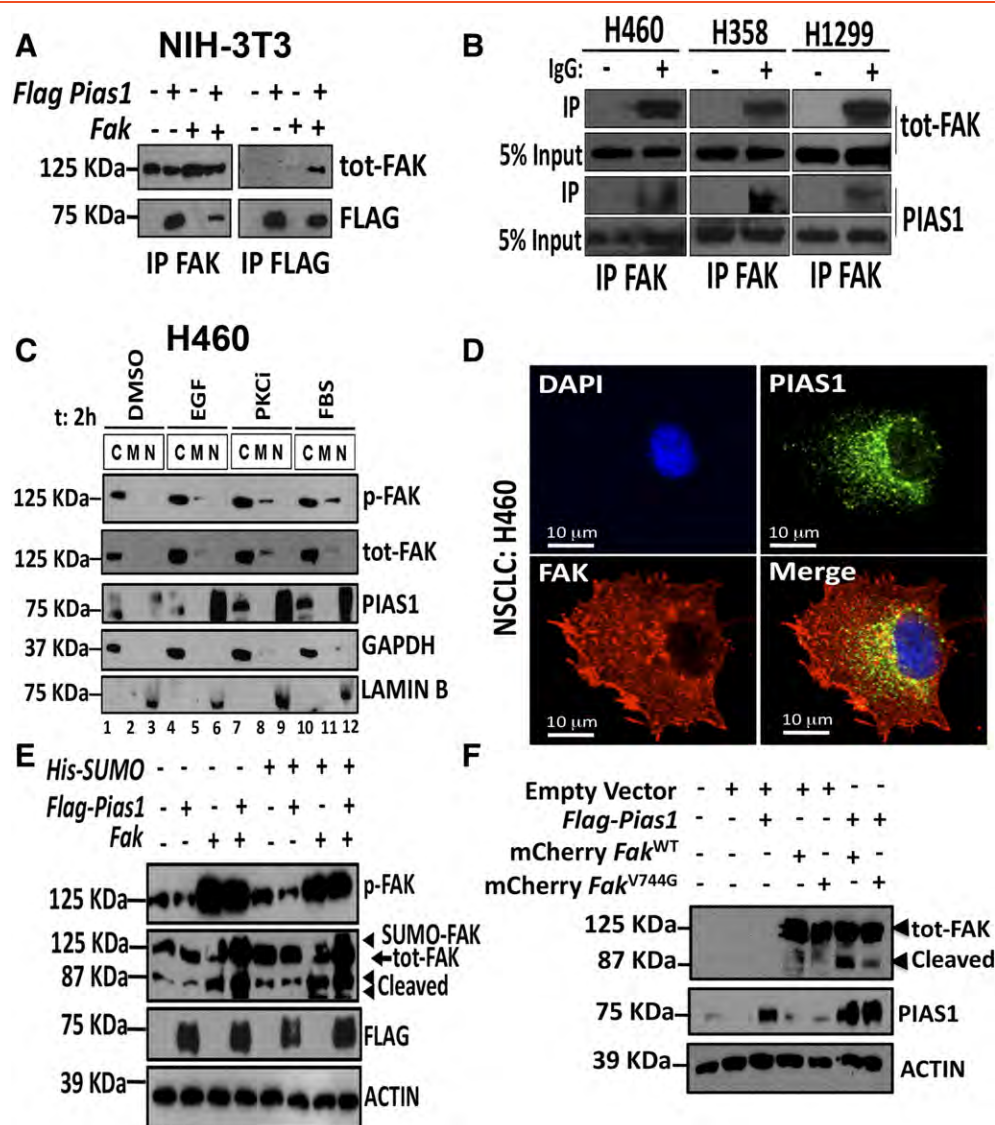


Figure 2. PIAS1 and FAK physically interact in human NSCLC cell lines. (A) NIH 3T3 cells were transiently transfected with the indicated plasmids and FAK and PIAS1 protein interaction was analyzed by co-IP followed by immunoblotting. (B) Immunoblot showing endogenous PIAS1 and FAK protein co-IP in the indicated NSCLC cell lines. (C) Subcellular fractionation and immunoblotting of endogenous FAK and PIAS1 proteins was performed in serum starved lung cancer cells H460 following treatment with: DMSO; EGF; protein kinase C inhibitor Calphostin (PKCi) and 10% FBS. Fractions: C; cytosolic; M; matrix; N: nuclear. Note significant cytoplasmic FAK and PIAS1 protein accumulation in PKCi and serum treatment, but not with EGF. (D) IF and confocal micrograph of FAK (red) and PIAS1 (green) localization at nuclear periphery in H460 NSCLC cells. Scale bar: 10 μ m. (E) Immunoblot following *Flag-Pias1* and *Fak* overexpression in NIH-3T3. Blot shows no change in total FAK or pFAK (Y397), but note the appearance of low and higher molecular weight species of FAK protein in cells overexpressing *Flag-Pias1* or *Flag-Pias1-SUMO1* overexpression, which correspond to SUMOylated-FAK and cleaved FAK. (F) Cells were transfected with *mCherry-Fak^{WT}* or *mCherry-Fak^{V744G}*, a cleavage resistant mutation, in combination with *Flag-Pias1* and analyzed with immunoblot as indicated. Note that *Flag-Pias1* promotes the cleavage of *mCherry-Fak^{WT}* as expected, but cleavage of C-terminus mutant *FAK^{V744G}* is impaired.

PIAS1 Silencing Impairs the Ability of NSCLC Cells to Form Colonies on Soft Agar

To elucidate how PIAS1-FAK interaction can modulate lung cancer progression we tested the effect of *PIAS1* inhibition on cell proliferation and survival. We found that *PIAS1* silencing with a short hairpin RNA (shRNA) had a modest effect on cell growth on plastic for most NSCLC cell lines (Figure 4A). Next, we examined how *PIAS1* inhibition would affect growth of NSCLC cells in soft agar. Indeed, following *PIAS1* inhibition, we observed a marked reduction in colony formation in soft agar assays (Figure 4, B–D).

Next we tested whether *PIAS1* inhibition may result in defective p-FAK activation, which could explain the inability of NSCLC cells with silenced *PIAS1* to establish colonies in soft agar assays. Surprisingly, p-FAK levels did not correlate with soft agar growth impairment in lung cancer cells (Figure 4E). Notably, the degree of knockdown achieved by shRNA A and B correlates with their effect on cell viability (Figure 4A). However, *PIAS1* silencing promotes increase baseline levels of pro-apoptotic protein BIM in several lung cancer cells including: H2228 and H1395 (Figure 4F). This finding suggests that *PIAS1* silencing does not directly affect p-FAK or

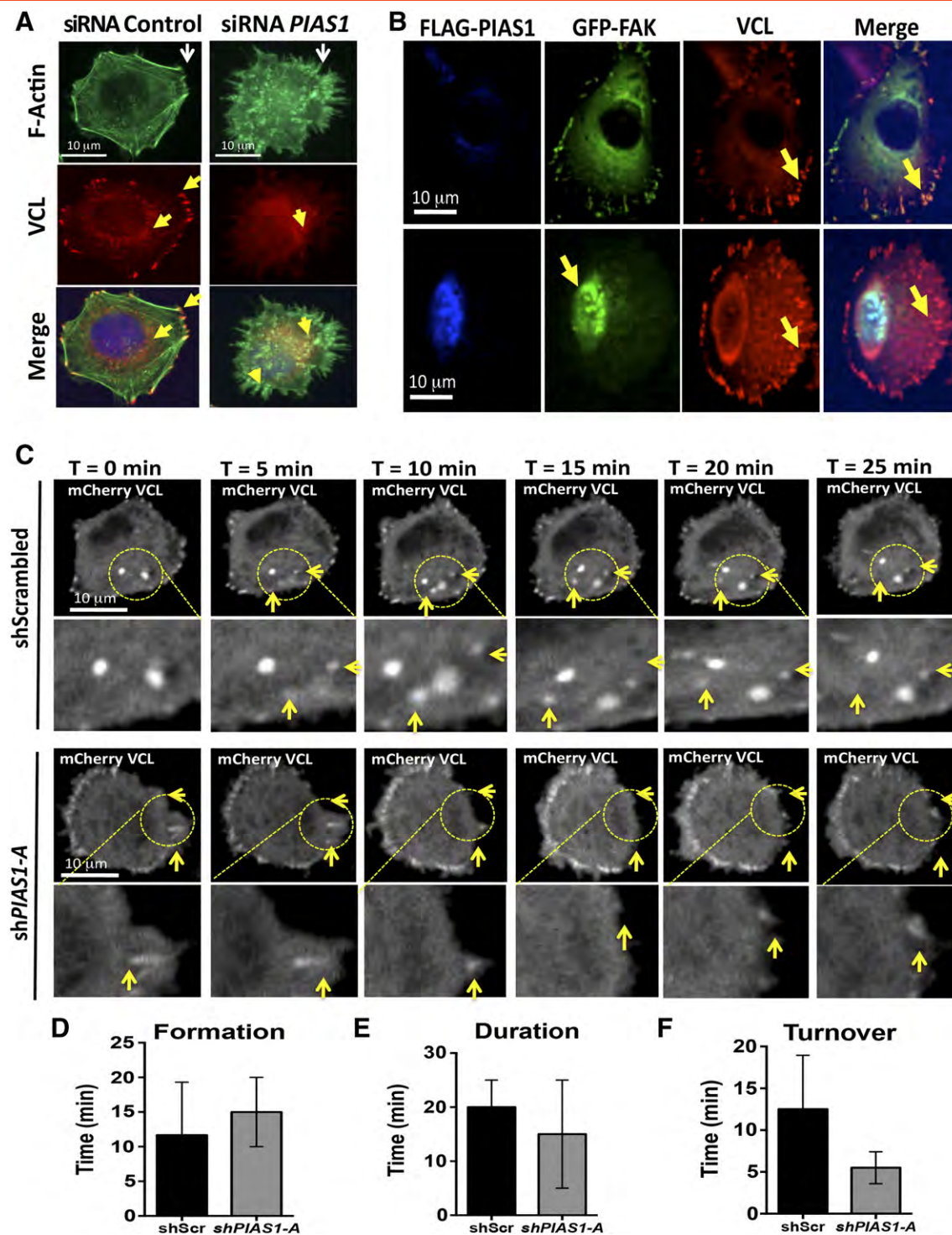


Figure 3. *PIAS1* silencing reduces stable stress fiber formation. (A) IF image of filamentous Actin (F-Actin) fiber formation in H460 NSCLC cells. White arrows indicate F-Actin fibers in control siRNA treated cells (Ctrl siRNA) or following *PIAS1* gene silencing; yellow arrows indicates Vinculin (VCL) protein localization to F-Actin fibers, which is lost in cells with *PIAS1* silencing. Scale bar: 10 μm. (B) IF image of VCL and GFP-FAK co-localization at F-Actin fibers in HBECs cells following ectopic *Flag-Pias1* expression. Top row shows HBECs cells expressing ectopic *GFP-Fak* and endogenous *VCL*. Bottom row image shows HBECs expressing ectopically expressed *Flag-Pias1* and GFP-FAK stained as indicated. Note FAK nuclear accumulation and increase in VCL puncta at focal adhesions (yellow arrows). Pink arrows indicate co-localization of GFP-FAK and VCL proteins. Scale bar: 10 μm. (C) Confocal live cell imaging of H460 NSCLC cells examining *mCherry-VCL* puncta formation dynamics in cells expressing the indicated shRNAs. Yellow arrows indicate sites of rapid focal adhesion turnover over a time lapse of 25 minutes. (D-F) Histograms show the average time for *mCherry-VCL* puncta formation, duration and turnover. Values represent an average of $n = 3$ cells.

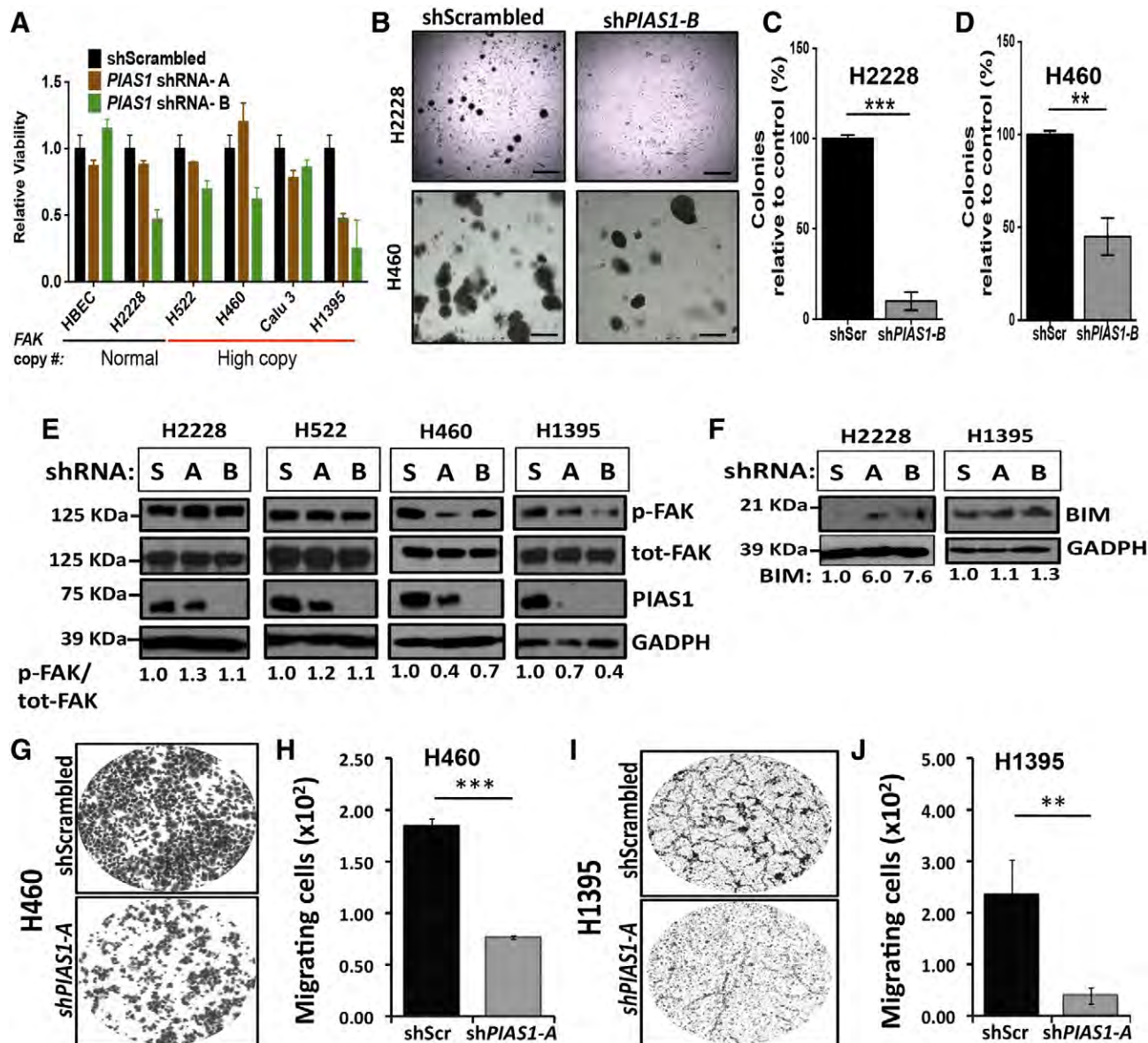


Figure 4. *PIAS1* silencing impairs tumor progression in NSCLC cells with *FAK* gene amplification. (A) Histogram shows cell viability following *PIAS1* gene silencing. *FAK* gene copy number status is also indicated. (B) Representative soft agar colonies of NSCLC cells expressing the indicated shRNA. Scale bar: 500 μ m. (C) Quantification of soft agar colonies of H2228 NSCLC cells 3 weeks post plating. (D) Quantification of soft agar colonies of H460 NSCLC cells 10 days post plating. (E) H2228, H522, H460 and H1395 NSCLC cells were treated with the indicated shRNAs and analyzed by Immunoblot with the indicated antibodies. (F) Immunoblot following *PIAS1* shRNA knockdown in H2228, H460 and H1395 NSCLC cells shows BIM pro-apoptotic protein upregulation. (G) Representative image of a Transwell migration assay performed with H460 cells expressing the indicated shRNAs. (H) Histogram shows the absolute number of migrating cells in triplicate wells following *PIAS1* knockdown in lung cancer cell line H460. Student's *t* test = ****P* < .001. (I) Representative image of a Transwell migration assay performed with H1395 cells expressing the indicated shRNAs. (J) Histogram shows the absolute number of migrating cells in triplicate wells following *PIAS1* knockdown in H1395 cells. Student's *t* test = ***P* < .01.

total-FAK protein levels in NSCLC, but promotes changes in FAK subcellular localization that are independent on FAK phosphorylation. Furthermore, these findings indicate that *PIAS1* silencing lowers the threshold for apoptosis in NSCLC cells.

Our analysis of human NSCLC samples suggested that *PIAS1* and *FAK* proteins are elevated in a subset of highly invasive tumors. Thus, we tested whether *PIAS1* silencing affects NSCLC cell invasion and migration potential *in vitro*. To do this, we performed Transwell migration and scratch assays in NSCLC cells with gene amplification of *FAK* and *PIAS1* [21,30,39]. In Transwell assays, H460 cells expressing the control shRNA completed migration after 16 hours (16 h) [40],

whereas cells expressing a shRNA targeting *PIAS1* showed reduced migration through Transwell membranes (Figure 4, G–H). These findings were replicated in H1395 cells, another NSCLC cell line with gene amplification of *FAK* and *PIAS1* (Figure 4, I–J).

These results suggest that *PIAS1* suppression significantly reduce the migration capacity of NSCLC cells. Thus, we performed scratch migration assays using NSCLC cells: H1792, H522 and H460 as representative examples of NSCLC cells from our panel. We found that *PIAS1* suppression did not have an effect on H1792, which has a loss of *FAK* gene copy number, but reduced the migration of H522 and H460, which have substantially higher levels of *FAK* and *PIAS1*

protein (Supplementary Figure 4, A–F). We assessed for changes in the cytoskeleton following scratch formation in H460 cells by staining for F-Actin and cells polarity with the GM130 Golgi maker. After 8 hours, cells transfected with the siRNA control were oriented towards the scratch site, whereas cells with *PIAS1* silencing were not, suggesting reduced ability to polarize towards the scratch site (Supplementary Figure 4, G–H).

In view of our findings we conclude that *PIAS1* suppression is associated with reduced anchorage-independent growth. In addition, *PIAS1* silencing reduces cell polarization during stimulus-driven migration in NSCLC cells with *FAK* and *PIAS1* gene amplification.

PIAS1-FAK Interaction Regulates Gene Transcription

We determined that *PIAS1* promotes focal adhesion maturation and FAK protein nuclear accumulation. This phenotype was conserved in NSCLC cells, NIH-3T3 fibroblasts and HBECs (Figure 5, A–B). GFP-FAK nuclear accumulation also occurs in HBECs cells harboring the oncogenic *KRAS*^{G12D} mutation and *p53* knockdown, suggesting a positive correlation with cancer progression (Figure 5B).

To test whether *PIAS1-FAK* interaction and FAK nuclear accumulation are directly associated with a pro-tumorigenic gene transcription program, we analyzed HBECs ectopically expressing *GFP-Fak* and *Flag-Pias1*. We analyzed mRNA-transcript linearized data with the Benjamini-Hochberg statistics (log ratio 0.6; and p value < 0.3) and subtracted values obtained from HBECs expressing *GFP-Fak* alone or *Flag-Pias1* alone to obtain transcripts that change only when both genes are co-expressed in HBECs. We identified 473 differentially up/down-regulated transcripts, which we used for further characterization (GEO accession ID: GSE73280). Using gene ontology analysis we uncovered that co-expression of *GFP-FAK* and *PIAS1* correlates with activation of several transcriptional programs that include DNA damage repair genes, oxidative phosphorylation genes and a pancreatic adenocarcinoma signature (Figure 5D). This finding suggests that nuclear FAK may participate in DNA repair and cell cycle progression, a hypothesis consistent with the known function of *PIAS1* in these processes [1,5].

To test whether *FAK* is involved in DNA repair or mitochondrial metabolism, we targeted its deletion in NSCLC cells by CRISPR/Cas9 gene editing. First, we tested for changes in γ H2AX and pCHK2, which are well-known DNA damage response genes [31,41]. We found that in addition to having baseline activation of γ H2AX and phospho-Chk2 (pCHK2), surrogate markers of DNA damage, *FAK* null (*FAK*[−]) H460 NSCLC cells were hypersensitive to ionizing radiation (IR) (Figure 5E). This result suggests a link between FAK nuclear localization and DNA damage response in NSCLC cells. We also tested the effect of *FAK* loss on oxidative phosphorylation by assessing ATP production and oxygen consumption rate. We found that in agreement with the perturbation in oxidative phosphorylation signatures during *PIAS1-FAK* overexpression, FAK protein depletion by CRISPR/Cas9 led to a reduced mitochondria ATP production and oxygen consumption rates (Figure 5, F and G).

Taken together these results suggest that *PIAS1*, by promoting FAK nuclear localization, promotes tumor progression by engaging transcriptional programs that regulate DNA damage repair, and oxidative phosphorylation.

PIAS1 Silencing is Detrimental for Xenograft Tumor Growth In Vivo.

Lastly, we examined whether *PIAS1* was required for tumor engraftment *in vivo* by performing xenograft experiments. We compared

H460 cells growth rate *in vivo* following stable viral transduction of control shRNA or a *PIAS1* shRNA. Xenografts expressing *PIAS1* shRNA showed a significant reduction in growth 15 days post implantation as compared to controls ($P < .05$) (Figure 6A). Furthermore, mice in the control group were euthanized on average after 37-days due to tumor burden; whereas the survival of mice in the *PIAS1* shRNA group averaged 55 days (Figure 6B). We performed postmortem histological analysis of xenograft tumors and found no significant difference in cell morphology or vascularity (Figure 6C). We then examined the contribution of shRNA harboring cells to the xenograft using the GFP reporter in the shRNA vector backbone and discovered a significant underrepresentation of GFP positive cells in the *PIAS1-shRNA* treatment group as compared to shRNA controls ($**P < .05$) (Figure 6, D–E).

In view of these findings, we concluded that *PIAS1* is required for the growth of tumor xenografts *in vivo*. Furthermore, *PIAS1* inhibition is selected against during tumor growth as demonstrated by the underrepresentation of *PIAS1* shRNA expressing cells at the experiment endpoint.

Discussion

Metastasis accounts for more than 90% of cancer related deaths worldwide [34,35,42,43]. Consequently, identification of novel biomarkers and potential therapeutic targets is of paramount importance. FAK nuclear localization has been reported to be important for disease progression in various cancer types and a requirement during embryogenesis and tissue homeostasis [40,44,45]. However, the mechanism that mediates FAK relocation to the cell nucleus and if and how this enhances cell survival, metastasis and tissue development has remained unclear [25,46].

Using SNPs data, we discovered that the SUMO-E3 ligase *PIAS1* and *FAK* genes are co-amplified in a subset of NSCLC specimens. Furthermore, we show a positive correlation between gene copy number gain and increase in total FAK and *PIAS1* protein levels, using NSCLC cell lines *in vitro* and human lung tumor samples *in vivo*. Interestingly, we found that FAK and *PIAS1* protein are elevated in a subset of primary and invasive human lung tissues and in a *bona fide* mouse model of NSCLC metastasis [35].

It was previously reported that FAK and *PIAS1* protein interact in a yeast two-hybrid screen and in transfected HEK-293T cells. In this setting *PIAS1* promotes FAK phosphorylation at tyrosine 397 (Y397), a key event for FAK activation [47]. We confirmed that endogenous *PIAS1* and FAK interact in NSCLC cells and this interaction is observed at the nuclear periphery or within the cell nucleus. However, our findings indicate that *PIAS1* expression does not affect P-FAK or total FAK protein levels in NSCLC, instead our data support the conclusion that *PIAS1* promotes changes in FAK subcellular localization that are independent on its phosphorylation. Indeed, *PIAS1* expression, or the combined expression of *PIAS1* and *SUMO-1*, results in the appearance of a ~87 KDa form of FAK, previously reported as a Calpain-mediated FAK cleavage product [38]. Indeed we found that using a point mutant form of FAK protein (*Fak*^{V74G}), that renders FAK resistant to Calpain proteolysis, the ~87KDa form of FAK was significantly reduced. Because FAK cleavage is part of its negative regulation at focal adhesions, we conclude that *PIAS1* may be involved in the regulation of FAK and focal adhesion dynamics.

Recently, inhibition of *PIAS1* was found to reduce breast cancer tumorigenesis by increasing genomic instability and loss of stem cell potential [7]. Silencing of *PIAS1* in a subset of NSCLC cells with *FAK*

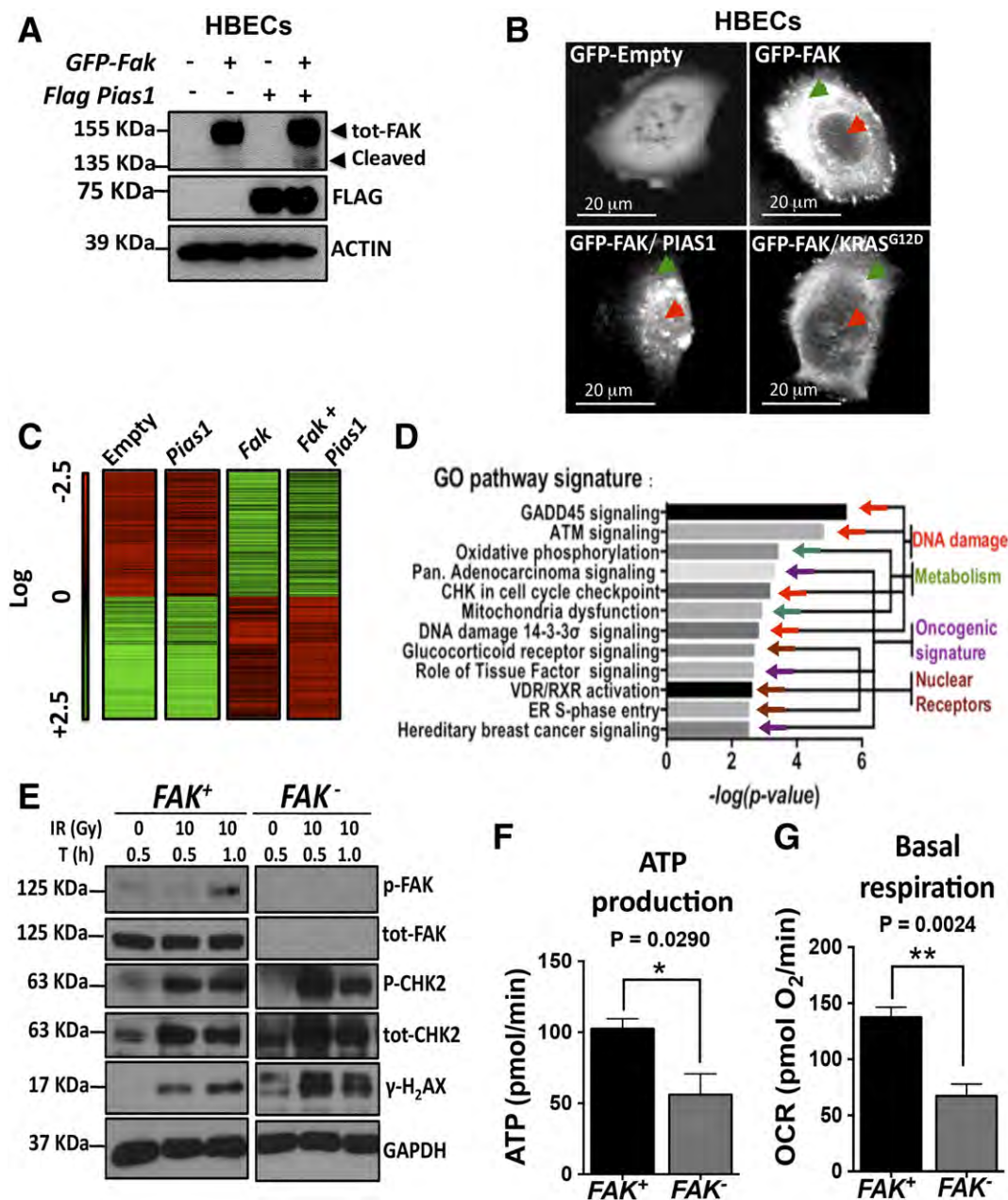


Figure 5. PIAS1 promotes FAK protein nuclear localization and gene transcription. (A) Immunoblot of immortalized HBECS transduced/transfected with the indicated plasmids. Arrow indicates cleaved FAK. (B) Confocal microscopy of HBECS transduced as indicated. Note the nuclear relocation of GFP-FAK after either ectopic *Flag-Pias1* overexpression in HBECS or cells harboring *KRAS*^{G12D} mutations (green and red arrows indicate cytoplasmic and nuclear FAK, respectively). Scale bar: 20 μ m. (C-D) Heatmap and gene ontology analysis of HBECS expressing *GFP-Fak* alone or in combination with *Flag-Pias1*. (E) Immunoblot of FAK wild type (FAK⁺) or FAK deleted (FAK⁻) H460 NSCLC cells treated with IR and harvested at the indicated time points. Note upregulation of p-CHK2 and γ -H₂AX in null cells, indicating DNA damage hypersensitivity following FAK loss. (F) Histogram shows the change in ATP productions (pmol/min) and oxygen consumption rate (OCR) in H460 NSCLC following FAK gene deletion. Analysis of mitochondrial function shows reduced ATP production and oxygen consumption in FAK⁻ H460 cells.

amplification results in deregulated F-Actin formation and increase in focal adhesion turnover. We found that *PIAS1* expression can promote GFP-FAK nuclear accumulation and rescues the accumulation of VCL puncta at focal adhesions. The latter correlates with an increase in the activation of integrin downstream targets (RAC-1 and ROCK-1), but not pFAK activation, as it is no longer at the cell membrane where its phosphorylation is known to occur [48,49].

Although FAK is widely associated with metastasis [10,11,25], it is still unclear whether PIAS1 or SUMOylation negates or contributes

to tumor progression [5,50]. PIAS1 was reported to repress TGF- β and reduced EMT transition in breast cancer cells by repression of *N-cadherin* [6]. However, other reports suggest that PIAS1 is necessary for breast cancer progression via activation of WNT5A, regulation of the estrogen receptor (ER) signaling and promoting tumor growth *in vivo* [7]. In our study, we found that *PIAS1* silencing in NSCLC did not have an effect on EMT genes including *N-Cadherin* or *E-Cadherin* (JDC and PPS, unpublished data). However, *PIAS1* silencing in NSCLC reduced cell polarization and

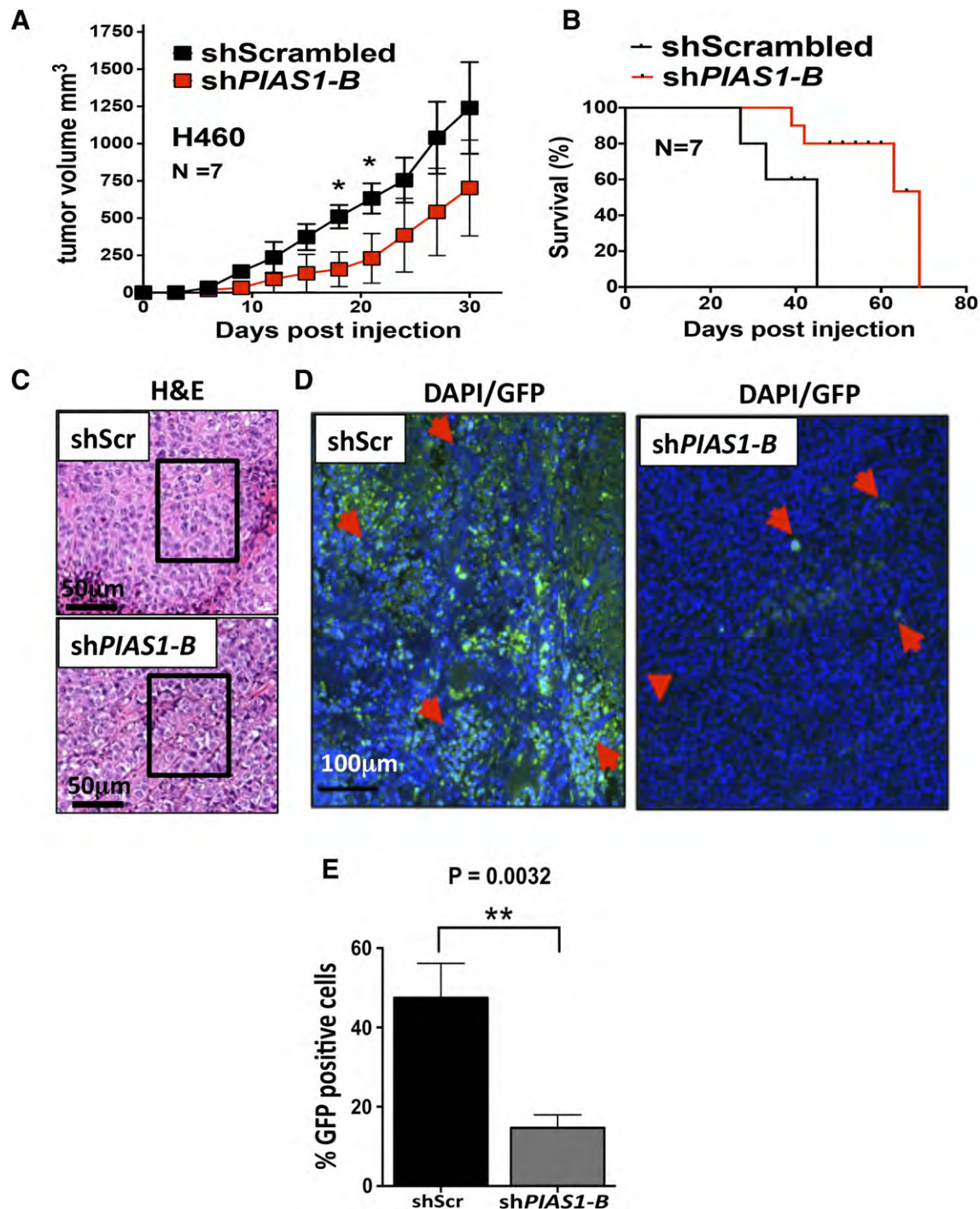


Figure 6. *PIAS1* gene silencing impairs xenograft tumor growth *in vivo*. (A) The histogram shows the volume of xenografts from H460 cells grown subcutaneously in NOD-SCID mice following transduction with scrambled shRNA (shScr; black line) or *PIAS1* shRNA (shPIAS1; red line). Mice per group N = 7. (B) Kaplan–Meier curve of mice carrying xenografts shown in panel A. Mice were sacrificed when the tumors reached 2000 mm³. (C) H&E histological analysis of xenografts expressing shScr or *PIAS1* shRNA at the end point of the experiment. Squares indicate the sections analyzed for the GFP reporter in the shRNA construct. (D) Anti-GFP IF shows loss of GFP positive cells in the *PIAS1* shRNA xenograft group compared to the scramble shRNA group. (E) Quantification of GFP positive cells in the indicated xenograft sections. Student's *t* test = ***P* < .01.

migration, suggesting that *PIAS1* contributes to NSCLC stimulus-driven migration. Specifically, we observed deficient lamellipodia formation and GM130 leading edge orientation following migration stimulus *in vitro*, which may have contributed to cells inability to integrate extracellular signaling and properly migrate. We propose

that FAK recruitment to the nucleus by means of increase interaction with *PIAS1*, allows for focal adhesion maturation and increase integrin signaling, as demonstrated by the appearance of lamellipodia projections in *Flag-Pias1* expressing cells. In contrast, lamellipodia are decreased or absent in migrating cells after *PIAS1* knockdown. *PIAS1*

gene silencing in NSCLC cells also reduced cell viability, independently of P-FAK activation. Following *PIAS1* gene silencing we observed a concomitant increase in BIM protein levels. In addition, we also observed a significant decrease in soft agar growth following *PIAS1* knockdown.

An unexpected result in our study was that PIAS1-induced FAK nuclear recruitment promotes DNA repair transcription program. Because FAK deletion led to DNA-damage hypersensitivity, shown by γ H2AX and pCHK2 activation, we speculate that the co-amplification of *FAK* and *PIAS1* promotes DNA damage repair, providing a survival advantage to genomically unstable tumors. In fact, FAK was found to be a negative regulator of p53 tumor suppressor in immortalized fibroblasts, and FAK inhibition radiosensitizes head and neck carcinoma [46,51]. Work is already in progress to characterize in better detail the involvement FAK in DNA damage repair and possible applications for NSCLC radiotherapy. Another unexpected finding was that silencing of *FAK* is associated with decrease of oxygen consumption and ATP production, an observation that underscores the importance of FAK in the maintenance of FAK-dependent NSCLC cells. Work is currently in progress to further characterize this phenotype.

In agreement with previous findings in breast cancer, *PIAS1* silencing affects NSCLC cells growth in soft agar *in vitro* and tumor xenograft growth *in vivo*. We speculate DNA damage regulation in NSCLC may provide the pro-survival signaling required for tumor progression in *PIAS1/FAK* overexpressing cancers. It will be of interest to identify the transcriptional modulators interacting with FAK while in the nucleus, as they represent potential tumor biomarkers or therapeutic targets.

Finally, our results show that a subset of NSCLCs has co-amplification of *FAK* and *PIAS1* and that these proteins are enriched in metastatic NSCLC. We conclude that PIAS1 is oncogenic and that, at least in part, this activity depends on its ability to promote FAK nuclear accumulation, integrin signaling activation and DNA damage repair. We conclude that the FAK-PIAS1 signaling axis is a novel regulator of NSCLC progression, integrating extracellular cues that regulate cell survival, migration and the DNA damage response. We propose that FAK-PIAS1 status would serve as a biomarker for the selection of patients undergoing personalized cancer treatment protocols likely to respond to FAK inhibitors currently in clinical trials.

Conflict of Interest

Authors declare no conflict of interest.

Author Contribution

JDC and PPS conceived the project, designed experiments and wrote the manuscript. JDC, KT, SM, HL and MM performed experiments; XT, JRC and IW performed and interpreted tumor microarray histochemistry experiments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neo.2016.03.003>.

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